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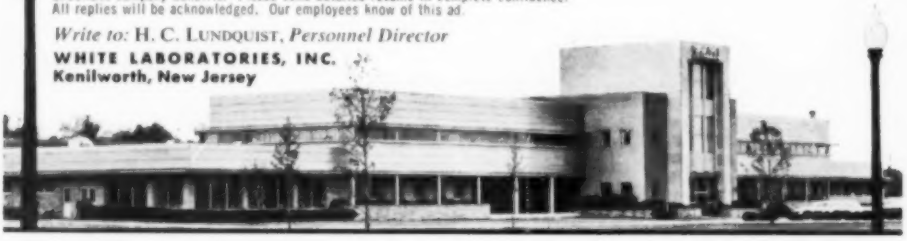


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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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# Enteric Coatings III*

## An Improved Enteric Coating and Its *In Vitro* Evaluation

By JOHN G. WAGNER and STUART LONG

**A new, improved enteric coating, prepared from styrene-maleic acid copolymer, dibutyl phthalate, and talc (I) was compared with previously reported coatings containing starch acetate phthalate, dibutyl phthalate, and talc (II), and cellulose acetate phthalate, propylene glycol, talc, and sorbitan monooleate (III). Increasing the amount of talc applied per tablet increases the disintegration time of the coated tablets in artificial intestinal fluid. This effect is least for I, intermediate for II, and greatest for III. A minimum amount of talc is essential to obtain satisfactory acid resistance with I but an increase in the amount of talc does not confer increased acid resistance. The ratio of gastric resistance time : disintegration time in artificial intestinal fluid is greater for I than for II. The ratio of disintegration time in artificial intestinal fluid : weight of enteric coating is much less for I than for either II or III. These observations clearly indicate advantages of I over II and III as enteric coatings. The types of plots presented are useful for comparing one type of coating with another and predicting the properties of the different coatings.**

VARIOUS DEFINITIONS of an efficient or suitable enteric coating have appeared in the literature (1, 2). It is proposed that the ideal enteric coating is one which would: (*a*) not disintegrate in the stomach in the duration of time that the enteric coated dosage form may be expected to remain in the stomach; (*b*) release the contained drug immediately upon reaching the upper small intestine; (*c*) have a disintegration time *in vitro* and *in vivo* in intestinal fluids which is essentially independent of the thickness or weight of the coating; (*d*) show no change in properties under normal or accelerated storage conditions; (*e*) be nontoxic; (*f*) be easy to apply; and (*g*) be economical. Such ideals are probably impossible to attain in practice but the closer a given coating approaches them, the better the coating.

From a practical standpoint it is more important to ensure that an enteric coated dosage form of many drugs will liberate the contained drug at the major site of absorption of these drugs, namely the duodenum and upper small intestine, than it is to ensure that the coating will remain intact in the stomach for many hours.

In this communication, the third in the series (3, 4), the *in vitro* evaluation of a new improved enteric coating is reported. This coating was recently disclosed (5). It was discovered that styrene-maleic acid copolymer forms excellent enteric films in combination with a micaceous mineral solid. Usually a plasticizer is also incorporated into the enteric film. Coatings prepared from styrene maleic acid (SY-MA) copolymer, di-*n*-butyl phthalate, and talc have excellent enteric properties in the dog[1] and human beings. Results obtained in human subjects will be reported in a subsequent communication. One of the more interesting aspects of this coating is its relatively high resistance *in vitro* up to pH 2.6 and its high susceptibility to breakdown at and above pH 3.4; this unique property will be discussed subsequently, also.

Very little investigation of the effect of dusting powders on the properties of enteric coatings has

* Received August 13, 1959, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

[1] Lots I and III of the previous publication (3) were the same as lots BI-1 and BIII-2, respectively, in this publication.

been reported in the literature. It seems to have always been assumed, until recently, that the powder was inert and contributed little to the disintegration behavior of the coating. Hawkins and Thompson (2) investigated the effect of a large number of dusting powders, each at one concentration level, with three different enteric coating solutions. Since only one concentration level was used in each case, it was impossible to determine what real effect each dusting powder had on the properties of each of the coating compositions. A British patent (6) disclosed that dispersion of a finely divided mineral solid in an enteric film-forming substance, such as cellulose acetate phthalate, rendered the coatings substantially free from cracking and crazing upon aging. We have found that the concentration level of talc in three modern enteric coatings is an important factor in determining the disintegration characteristics of the resultant coatings.

Reports concerning enteric coatings in the past have always stressed the differences in results achieved with the various enteric substances. In this communication it is stressed that one should not compare enteric substances unless one takes into consideration the amounts of the enteric substance and the dusting powder employed. Also certain empirical relationships which are useful in predicting the properties of enteric coatings and in comparing one type of coating with another are presented.

## EXPERIMENTAL

**Materials.**—The styrene-maleic acid copolymers used were obtained by complete hydrolysis of styrene-maleic anhydride copolymers. The latter were prepared by conventional polymerization techniques (7, 8, 9). The type 1 copolymer had a considerably greater molecular weight than the type 2 copolymer. Further details may be found in reference (5). The starch acetate phthalate was S.A.P. III described in the previous publication (4) of this series. The cellulose acetate phthalate was described previously (4). Other materials were: di-*n*-butyl phthalate #1403 (Eastman Organic Chemicals), methyl ethyl ketone (Shell Development Corp.), and U. S. P. supreme talc (Sierra Talc and Clay Co.).

**Enteric Coating.**—The properties of the compressed and subcoated tablets prior to enteric coating are shown in Table I. The subcoated tablets were coated with different enteric coating solutions (see Table II) by conventional pan coating techniques. In most cases talc was used as a dusting powder.

In study A, two lots of subcoated tablets were enteric coated with solution AI and two lots of the same subcoated tablets were enteric coated with solution AII. Talc was used as a dusting powder on one lot coated with solution AI and on one lot coated AII. No dusting powder was applied to the second lots coated with solutions AI and AII. The tablets of all four lots were coated with an average of 30.9 mg. of SY-MA copolymer per tablet. For the lots coated with talc and solutions AI and AII, the average weight of the enteric coating per tablet was 125 and 127 mg., respectively.

In study B, lots of subcoated tablets were coated with coating solutions BI and BII shown in Table II and with solution BIII, containing cellulose acetate phthalate. Solution BIII was prepared according to example 1 of reference (6). In study B the weight of enteric polymer applied per tablet was held essentially constant but the amount of dusting powder applied per tablet varied from lot to lot. Lots BI-1, BI-2, BII-1, and BII-2 were all coated with an average of 25 mg. of styrene-maleic acid copolymer per tablet. The weight of talc applied per tablet, however, varied from 91 to 147 mg. Lots BIII-1, -2, and -3 were coated with approximately the same amount of cellulose acetate phthalate per tablet, namely 13.5 ± 1.5 mg. The weight of talc applied per tablet was varied from 49 to 88.7 mg.

In study C, lots of 10,000 subcoated tablets were coated with coating solutions CI, CII, and CIII (see Table II). After a definite amount of enteric coating had been applied some of the tablets of each batch were withdrawn and dried. In study C both the weight of enteric polymer and the weight of talc applied was increased from lot to lot for a given enteric polymer but the ratio of talc:enteric polymer remained essentially constant. For lots CI-1 through CI-6 this ratio averaged 3.09, with a range of 2.91 to 3.21. For lots CII-1 through CII-6 this ratio averaged 2.85, with a range of 2.55 to 3.05. For lots CIII-1 through CIII-5 this ratio averaged 2.71 with a range of 2.55 to 2.79.

**Enteric Coated Tablets.**—The resistances of the four lots of enteric coated tablets, which were coated in study A, to simulated gastric fluid U. S. P. and their disintegration times in artificial intestinal fluid, pH 6.9, are shown in Table III.

Tables IV and V give the properties of the enteric coatings produced in studies B and C, respectively. The thicknesses of the enteric coatings on the side and edge of the tablets of lots CI-1 through CI-6 are plotted in Fig. 7. These thicknesses were determined by individually measuring 100 enteric coated and 100 subcoated tablets of each lot with a micrometer reading to 0.001 inch.

*In vitro* disintegration tests were carried out using the apparatus, fluids, and end points described in a previous publication (3). In study C, some of the lots were tested in artificial intestinal fluid, pH 6.9 by two methods: (*a*) by placing the tablets directly into fluid, and (*b*) by putting the tablets in simulated gastric fluid U. S. P. for two hours then transferring them to the other fluid. As before (3) the average disintegration time of each enteric coating, $T_c$, in the pH 6.9 fluid was assumed to be the difference in the average disintegration time of the whole enteric coated tablet and the average disintegration time of the corresponding subcoated tablet. The average time to the coating attacked end point in simulated gastric fluid U. S. P., $Tg$, was also determined as described previously (3). The standard errors, $S_{Ti}$, $S_{Tc}$, and $S_{Tg}$ of the averages $T_i$, $T_c$, and $T_g$, respectively, are also given in Tables IV and V.

TABLE I.—PROPERTIES OF THE COMPRESSED AND SUBCOATED TABLETS PRIOR TO ENTERIC COATING

| Lot of Enteric Coated Tablets | Compressed Tablet | | Subcoated Tablet[a] | | | | |
|---|---|---|---|---|---|---|---|
| | Av. Wt., Gm. | Punch Size and Shape, in. | Av. Thickness, in. Edge | Av. Thickness, in. Side | Av. Wt., Gm. | Av. Vol. cu. mm. | Av. Disintegration Time[b] ± S.E.[c] Min. |
| CI-1 to -6; CII-1 to -6; CIII-1 to -5 | 0.418 | 3/8 oval | 0.414 | 0.211 | 0.576 | ... | 7.4 ± 0.6 |
| BI-1 and -2; BII-1 and -2 | 0.426 | 3/8 oval | 0.407 | 0.207 | 0.542 | 301 | 4.8 ± 0.2 |
| BIII-1 | 0.266 | 3/8 oval | 0.405 | 0.199 | 0.376 | ... | 2.0 |
| BIII-2 and -3 | 0.437 | 3/8 oval | 0.403 | 0.214 | 0.543 | 305 | 8.8 ± 0.3 |

[a] The subcoatings were of the carboxymethyl cellulose type described in U. S. pat. 2,693,437 (1954).
[b] Average disintegration time of the subcoated tablets in artificial intestinal fluid, pH 6.9, using the U. S. P. apparatus. The end point was taken when 99 to 100% of the entire tablet was through the screen of the disintegration apparatus.
[c] Standard error of the average.

TABLE II.—COMPOSITION OF COATING SOLUTIONS

| Ingredient | Solution[a] AI | AII | BI | BII | CI | CII | CIII |
|---|---|---|---|---|---|---|---|
| Styrene-maleic acid copolymer (type 1) | ... | ... | 15.625 | 15.625 | ... | 20 | ... |
| Styrene-maleic acid copolymer (type 2) | 5 | 15 | ... | ... | 20 | ... | ... |
| Starch acetate phthalate | ... | ... | ... | ... | ... | ... | 12.5 |
| Di-*n*-butyl phthalate | 0.6 | 1.8 | 1.875 | 1.875 | 2.4 | 2.4 | 1.5 |
| Alcohol 3A, *q. s.* | √ | √ | √ | ... | √ | √ | ... |
| Equal parts of alcohol 3A and acetone, *q. s.* | ... | ... | ... | √ | ... | ... | ... |
| Methyl ethyl ketone, *q. s.* | ... | ... | ... | ... | ... | ... | √ |

[a] Concentration expressed as % w/v. Check marks indicate solvent used for particular solution.

TABLE III.—DISINTEGRATION BEHAVIOR OF TABLETS PRODUCED IN STUDY A

| | Disintegration Tests, Time in Minutes | | |
|---|---|---|---|
| | $T_i$[a] ± $ST_i$ | $T_c$ | $T_g$ ± $ST_g$ |
| Compressed tablet | 0.5 ± 0 | ... | 0.4 ± 0[a] |
| Subcoated tablet | 4.5 ± 0.18 | 4.0 | 2.5 ± 0.19[a] |
| Enteric coated tablet (solution AI; no talc) | 9.25 ± 0.62 | 4.75 | 12.8 ± 1.94[a] |
| Enteric coated tablet (solution AI + talc) | 17.4 ± 1.00 | 12.9 | 238 ± 40[a] (180 ± 5.7)[b]; >233 [a] (>202)[b] [c] |
| Enteric coated tablet (solution AII; no talc) | 8.75 ± 0.44 | 4.25 | 11.6 ± 0.92[a] |
| Enteric coated tablet (solution AII + talc) | 17.7 ± 0.36 | 13.2 | 251 ± 16[a] (204 ± 10)[b]; >300 [a] (255 ± 24)[b] [c] |

[a] End point taken when 99 to 100% of tablet was through the screen of U. S. P. disintegration apparatus.
[b] Coating slightly attacked end point.
[c] Two separate tests. One test was terminated at six hours and since one or more tablets had not reached the end point the true average could not be computed.

**Evaluation of Enteric Coatings.**—Adequate evaluation of an enteric coating necessitates both *in vitro* and *in vivo* tests. Interpretation of the effect of different variables on the *in vitro* disintegration behavior of an enteric coating and comparison of different enteric coatings may conveniently be made by use of such plots as shown in Figs. 1 through 6. For a wide variety of enteric coatings the points on such plots can be fitted by the equations of the straight line, $Y = ax + b$ or $Y = ax$ or by a simple polynomial of the type $Y = ax + bx^2$. The slopes of the lines and their position characterize a given type of coating. Direct comparison of two different coating systems may also be made by comparison of the slopes of the lines obtained for these different systems on the same type of plot.

Values of disintegration time are slightly lower when the tablets are placed directly into artificial intestinal fluid than when they are tested in the same fluid following two hours in simulated gastric fluid. Practically, the difference is insignificant and in most cases a significant difference cannot be shown between two such individual values at a high level of confidence. However, when plotting the data the difference is important. When values of disintegration time in artificial intestinal fluid following gastric fluid are plotted against such abscissas as average weight of enteric coating, average weight of enteric polymer, etc., the lines obtained cannot be extrapolated to pass through the origin. Usually such lines have a negative intercept (e. g., see Fig. 1). However, when the average disintegration times obtained by direct immersion in artificial intestinal fluid are plotted against similar abscissas, lines are obtained which do pass through the origin when extrapolated (see Figs. 3 through 6).

## DISCUSSION

Three separate but interrelated studies are reported in this communication.

Study A compared coatings prepared from SY-MA copolymer and dibutyl phthalate, with and without talc, at one level of SY-MA copolymer and one level of talc. The enteric polymer was applied to the tablets as solutions of two different concentrations.

Study B compared coatings prepared from SY-MA copolymer and cellulose acetate phthalate. For each enteric polymer lots of tablets were coated with constant amounts of the enteric substance but

TABLE IV.—PROPERTIES OF ENTERIC COATINGS PRODUCED IN STUDY B

| Lot | Average Weight Applied/Tablet, mg. Enteric Polymer | Talc | Average Amount of Coating ± Standard Error Volume, cu. mm. | Weight,[a] mg. | Thickness,[a] Inches × $10^3$ Edge | Side | Disintegration Tests Intestinal, pH 6.9 After Gastric $Ti \pm St_i$ | $Tc$ | Simulated Gastric Fluid, pH 1.2 $Tg \pm St_g$ |
|---|---|---|---|---|---|---|---|---|---|
| BI-1 | 25 | 91 | 40.0 ± 1.2 | 81 ± 3.5 | 8.8 ± 0.8 | 7.6 ± 0.5 | 15.1 ± 0.3 | 10.3 | 285 ± 26 |
| BII-1 | 25 | 130 | 58.4 ± 3.1 | 138 ± 3.5 | 10.1 ± 0.5 | 10.9 ± 0.3 | 41.0 ± 0.4 | 36.2 | 232.5 ± 6.6 |
| BII-2 | 25 | 129 | 65.0 ± 2.2 | 138 ± 3.1 | 10.1 ± 0.5 | 11.2 ± 0.4 | 41.3 ± 0.7 | 36.5 | 235 ± 6.1 |
| BI-2 | 25 | 147 | 67.5 ± 1.9 | 150 ± 3.5 | 10.8 ± 0.4 | 11.5 ± 0.3 | 46.8 ± 0.3 | 42.0 | 225 ± 10.0 |
| BIII-1 | 13.1 | 49 | ... | 45 ± 2.1 | 3.7 ± 0.2 | 3.3 ± 0.5 | 14.8 ± 0.5 | 12.8 | ... |
| BIII-2 | 12 | 57.4 | 34.2 ± 1.5 | 73 ± 3.0 | 4.6 ± 0.3 | 6.3 ± 0.4 | 43.8 ± 0.6 | 35.0 | >420 |
| BIII-3 | 15 | 88.7 | 40.0 | 91 ± 3.1 | 6.5 ± 0.5 | 7.5 ± 1.0 | 60.0 | 51.2 | ... |

[a] Based on 20 individually measured enteric coated and subcoated tablets.

TABLE V.—PROPERTIES OF ENTERIC COATINGS PRODUCED IN STUDY C

| Lot | Av. Wt. Applied Per Tablet, mg. Enteric Polymer | Talc | Av. Wt. of Coating[a] mg. | Disintegration Tests, Time in Minutes Intestinal, pH 6.9 After Gastric $Ti \pm St_i$ | $Tc \pm St_c$ | Intestinal, pH 6.9 Directly $Ti \pm St_i$ | $Tc \pm St_c$ | Sim. Gastric Fluid, pH 1.2 $Tg \pm St_g$ |
|---|---|---|---|---|---|---|---|---|
| CI-1 | 10 | 32.1 | 33 | ... | ... | 11.8 ± 0.4 | 4.4 ± 0.4 | 68 ± 2.3 |
| CI-2 | 15 | 46.4 | 47 | ... | ... | 13.6 ± 0.2 | 6.2 ± 0.3 | 110 ± 2.3 |
| CI-3 | 19.7 | 59.8 | 60 | 14.8 ± 0.4 | 7.4 ± 0.4 | 14.2 ± 0.3 | 6.8 ± 0.3 | 125 ± 1.6 |
| CI-4 | 24.7 | 77.8 | 75 | 17.3 ± 0.5 | 9.9 ± 0.5 | 16.2 ± 0.2 | 8.8 ± 0.3 | 158 ± 4.2 |
| CI-5 | 29.7 | 86.4 | 84 | 19.1 ± 0.4 | 11.7 ± 0.5 | 18.0 ± 0.4 | 10.6 ± 0.5 | 160 ± 5.8 |
| CI-6 | 38.1 | 120.1 | 113 | 23.9 ± 0.2 | 16.5 ± 0.3 | 21.3 ± 0.2 | 13.9 ± 0.3 | 243 ± 7.4 |
| CII-1 | 9.97 | 29.8 | 33.9 | ... | ... | 12.1 ± 0.2 | 4.7 ± 0.3 | 82 ± 2.1 |
| CII-2 | 14.95 | 45.3 | 54.1 | ... | ... | 15.4 ± 0.4 | 8.0 ± 0.4 | 128 ± 3.6 |
| CII-3 | 19.93 | 56.1 | 70.1 | ... | ... | 16.9 ± 0.3 | 9.5 ± 0.3 | 120 ± 4.3 |
| CII-4 | 24.90 | 68.4 | 83.7 | 20.6 ± 0.4 | 13.2 ± 0.5 | 21.0 ± 0.3 | 13.6 ± 0.3 | 165 ± 8.2 |
| CII-5 | 29.83 | 79.8 | 96.7 | 24.0 ± 0.4 | 16.6 ± 0.4 | 23.8 ± 0.2 | 16.4 ± 0.3 | 193 ± 8.6 |
| CII-6 | 38.25 | 116.7 | 133.3 | 29.0 ± 0.4 | 21.6 ± 0.4 | 27.9 ± 1.1 | 22.5 ± 0.4 | 273 ± 12.1 |
| CIII-1 | 10 | 27.9 | 32.0 | ... | ... | 29.0 ± 1.1 | 21.6 ± 1.1 | 142 ± 13.5 |
| CIII-2 | 15 | 41.5 | 47.5 | 41.1 ± 1.5 | 33.7 ± 1.5 | 41.0 ± 1.3 | 33.6 ± 1.3 | 217 ± 7.8 |
| CIII-3 | 20 | 53.8 | 61.5 | 55.0 ± 1.7 | 47.6 ± 1.7 | 46.6 ± 2.0 | 39.2 ± 2.0 | 295 ± 8.6 |
| CIII-4 | 25 | 64.4 | 73.9 | 61.7 ± 1.8 | 54.3 ± 1.8 | 59.3 ± 3.0 | 51.9 ± 3.1 | 397 ± 11.8 |
| CIII-5 | 30 | 82.2 | 91.5 | 70.7 ± 1.5 | 63.3 ± 1.5 | 70.5 ± 1.8 | 63.1 ± 1.8 | 518 ± 15.0 |

[a] The weight of coating is the difference in weight of 500 enteric coated and 500 subcoated tablets.

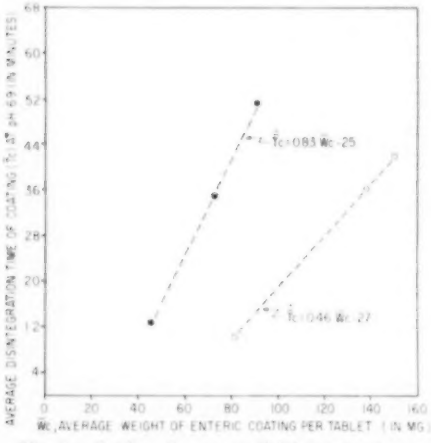


Fig. 1.—A plot of average disintegration time of the enteric coating in artificial intestinal fluid, pH 6.9 ($Tc$, in minutes) against the average weight of enteric coating per tablet ($Wc$, in milligrams). Coatings: ● - - - ● cellulose acetate phthalate, Span 80, propylene glycol, and talc. (Lots BIII-1, -2, and -3); ○ - - - - - ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots BI-1, -2, and BII-1, -2.)

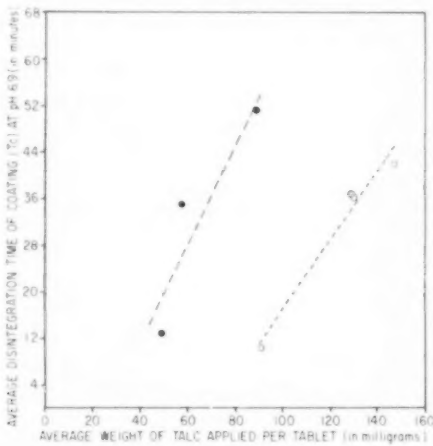


Fig. 2.—A plot of average disintegration time of the enteric coating ($Tc$, in minutes) against the average weight of talc applied per tablet (in milligrams). Coatings: ● - - - ● cellulose acetate phthalate, Span 80, propylene glycol, and talc. (Lots BIII-1, -2, and -3); ○ - - - - ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots BI-1, -2, and BII-1, -2.)

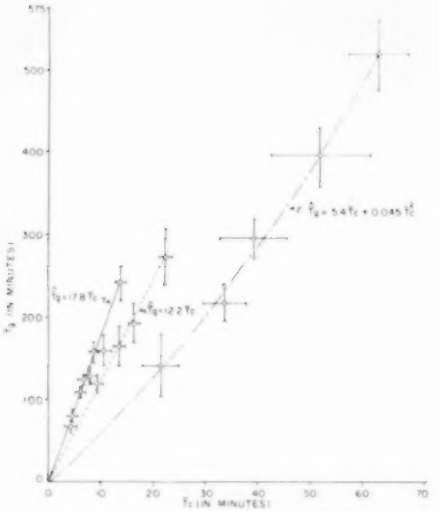


Fig. 3.—A plot of time (in minutes) to the coating attacked end point in simulated gastric fluid U. S. P. ($Tg$) against average disintegration time of the enteric coating ($Tc$, in minutes) in artificial intestinal fluid, pH 6.9, when the tablets were placed directly into the latter fluid. Coatings: ○——○ SY-MA copolymer (type 2), dibutyl phthalate, and talc. (Lots CI-1 through -6); ○ - - - - ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots CII-1 through -6); ○ — - — - ○ starch acetate phthalate, dibutyl phthalate, and talc. (Lots CIII-1 through -5.) Vertical and horizontal bars are 99% confidence intervals about the mean ordinate and abscissa values, respectively.

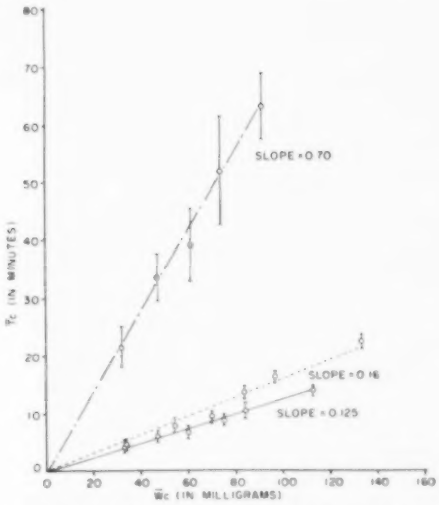


Fig. 4.—A plot of average disintegration time of the enteric coating ($Tc$, in minutes) when the tablets were placed directly into artificial intestinal fluid, pH 6.9, against the average weight of enteric coating per tablet ($Wc$ in milligrams). Coatings: ○ - ——- ○ starch acetate phthalate, dibutyl phthalate, and talc. (Lots CIII-1 through -5); ○ - - - - - ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots CII-1 through -6); ○ ——— ○ SY-MA copolymer (type 2), dibutyl phthalate, and talc. (Lots CI-1 through -6.) Vertical bars are 99% confidence intervals about the ordinate values.

different amounts of talc so that in each case the ratio of talc:enteric polymer varied from lot to lot.

Study C compared coatings prepared from SY-MA copolymer and starch acetate phthalate. In both cases dibutyl phthalate was used as plasticizer. For each enteric polymer, lots of tablets were coated with variable amounts of enteric polymer and talc but the ratio of talc to enteric polymer remained essentially constant from lot to lot.

In order to completely elucidate the relative effects of talc and enteric substance on the disintegration behavior of the resultant coatings it would be necessary to do a fourth type of study in which constant amounts of talc but variable amounts of enteric polymer were applied to a series of lots of tablets. In practice this type of study is the most difficult to perform due to the nature of the pan coating process.

Results of studies A and B indicate that not only is the dusting powder important from the standpoint of the process of enteric coating in the pan and the production of smooth coatings, but also the presence or absence and the amount of dusting powder used with a given enteric polymer largely determines the disintegration behavior of the coatings. Changing the talc:enteric polymer ratio or changing the amounts of dusting powder and polymer per tablet (keeping the ratio constant) changes the disintegration time of the coatings to a different degree for the various coating systems.

Figures 1 and 2 (derived from data of study B, Table IV) indicate that the amount of talc applied per tablet has a marked effect on the disintegration time of both types of coatings in artificial intestinal fluid. From these and similar plots it has been found that the effect of talc on disintegration time seems to be least for SY-MA copolymer coatings, intermediate for starch acetate phthalate coatings, and greatest for cellulose acetate phthalate coatings. However, resistance to simulated gastric fluid is largely determined by the amount of enteric polymer applied per tablet. Incorporation of a minimum amount of the dusting powder into the enteric film usually is essential to get satisfactory acid resistance but an increase in the amount of the dusting powder does not confer increased acid resistance. Because of the variable loss of dusting powder from lot to lot, plots based on the weight of coating, like Fig. 1, usually are more useful for predicting than plots based on weight of powder applied, like Fig. 2. If one assumed that no enteric polymer or plasticizer was lost in the coating pan (or the tablets were assayed for enteric polymer and plasticizer) then a more accurate plot of the type shown in Fig. 2 could be constructed.

Some of the variables involved in study C are plotted in Figs. 3 through 7. These plots are valid only for the ratios of talc:polymer employed.

When interpreting a plot of the type shown as Fig. 6 one must remember that the average talc applied:polymer applied ratio is 3.09 and that the

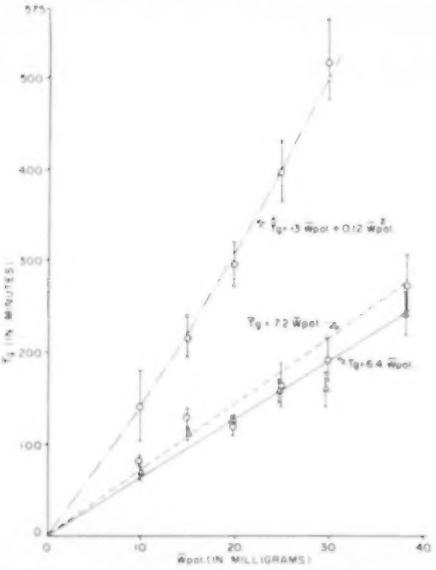


Fig. 5.—A plot of time (in minutes) to the coating and end point in simulated gastric fluid U. S. P. ($Tg$) against the mean weight of enteric polymer applied per tablet ($\bar{W}_{pol.}$, in milligrams). Coatings: ○ - ——— - ○ starch acetate phthalate, dibutyl phthalate, and talc. (Lots CIII-1 through -5); ○ - - - - ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots CII-1 through -6); △ ——— △ SY-MA copolymer (type 2), dibutyl phthalate, and talc. (Lots CI-1 through -6.) Vertical bars are 99% confidence intervals about the ordinate values.

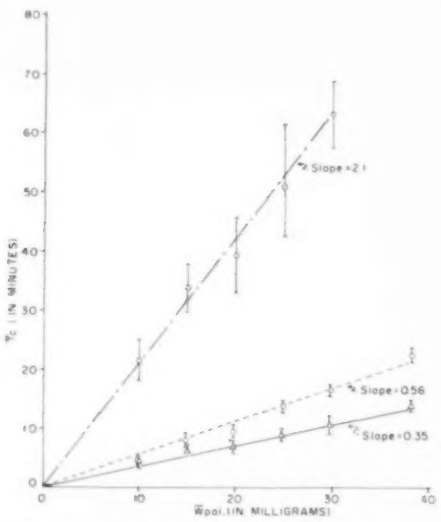


Fig. 6.—A plot of average disintegration time of enteric coating ($Tc$, in minutes) when the tablets were placed directly into artificial intestinal fluid, pH 6.9, against the average weight of enteric polymer applied per tablet ($\bar{W}_{pol.}$, in milligrams). Coatings: ○ - ——— - ○ starch acetate phthalate, dibutyl phthalate, and talc. (Lots CIII-1 through -5); ○ - - - - ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots CII-1 through -6); △ ——— △ SY-MA copolymer (type 2), dibutyl phthalate, and talc. (Lots CI-1 through -6.) Vertical bars are 99% confidence intervals about the ordinate values.

talc as well as polymer at each level is influencing the intestinal disintegration time of the coating.

The type of plot shown in Fig. 3 is an important one for comparison of different enteric coatings. Figure 3 illustrates that the rate of increase of gastric resistance in relation to increase in intestinal disintegration time is greater for the SY-MA copolymer coatings than for the starch acetate phthalate coatings. Comparison of the slopes of the lines at any given $Tc$ value can be made by differentiating the equation $\hat{T}g = 5.4\ Tc + 0.045\ Tc^2$ for the starch acetate phthalate coatings as follows:

$$\frac{d\,\hat{T}g}{d\,Tc} = 5.4 + 0.09\ Tc$$

When $Tc = 10$ minutes, the instantaneous slope is 6.3 and when $Tc = 20$ minutes, the slope is 7.2. Over the range studied the slopes for the SY-MA copolymer coatings, namely 12.2 and 17.8 are always greater than the instantaneous slope of the line for the starch acetate phthalate coatings. There is no doubt that the actual values of gastric resistance ($Tg$) for a given weight of enteric polymer applied are higher for both the cellulose acetate phthalate and starch acetate phthalate coatings than for the SY-MA copolymer coatings. This is readily seen in Fig. 5. However, it has been shown in dogs (3) that the *in vitro* gastric resistance test is not as reliable an index of *in vivo* resistance to stomach conditions as the *in vitro* intestinal disintegration test is an index to *in vivo* intestinal disintegration time. Tablets which withstand simulated gastric fluid for from one to four hours may exhibit much greater resistance to stomach conditions in the dog. Two other factors are: (*a*) it is only necessary to protect the enteric coated dosage form in the stomach for the duration of time that it may be expected to remain in the stomach; and (*b*) with many drugs it is more important to ensure that the enteric coated dosage form will liberate the contained drug in the intestines than it is to ensure that it will remain intact in the stomach for many hours.

Figures 1, 4, and 6 illustrate that SY-MA copolymer coatings exhibit the lowest dependence of disintegration time in artificial intestinal fluid on weight of enteric coating or weight of enteric polymer applied when compared to starch acetate phthalate or cellulose acetate phthalate coatings. This is extremely important from a manufacturing viewpoint. If the tablet coater inadvertently applies more than the usual amount of enteric coating the effect on intestinal disintegration time will be much less for SY-MA copolymer coatings than for either starch acetate phthalate or cellulose acetate phthalate coatings.

There has been speculation during the coating of tablets punched with a standard oval punch concerning which part of the coating builds up

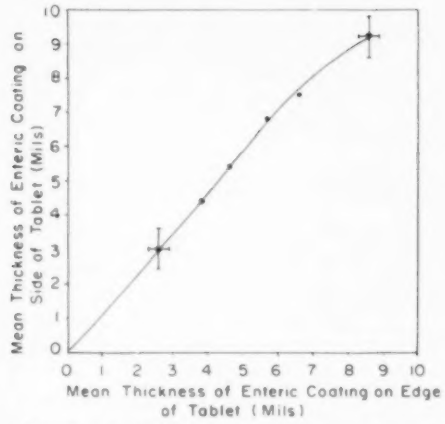


Fig. 7.—A plot of average thickness of enteric coating on the side of the tablet (in mils) against average thickness of enteric coating on the edge of the tablet (in mils). Tablets coated with SY-MA copolymer (type 2), dibutyl phthalate, and talc. (Lots CI-1 through -6.) Vertical and horizontal bars are 99% confidence intervals about the mean ordinate and abscissa values, respectively.

faster. Figure 7 shows a plot of average thickness of enteric coating on the side of the tablet (i. e., dimension of thickness of the tablet) against average thickness of enteric coating on the edge of the tablet (i. e., dimension of diameter of the tablet). This curve has a sigmoidlike shape, at first curving slightly upwards then reaching an inflection point, then curving downwards. Hence in this case, the coating on the side of the tablet built up faster than coating on the edge at first; then a point was reached where the build-up was equal for both dimensions; and finally, the coating on the edge built up faster than the coating on the side of the tablet.

## SUMMARY AND CONCLUSIONS

1. The effect of some of the variables in the enteric coating process on the disintegration behavior of three modern enteric coatings was elucidated.

2. Some of the properties of a new improved enteric coating were reported. The new coating is prepared from styrene-maleic acid copolymer, dibutyl phthalate, and talc.

3. Certain empirical relationships which are useful in predicting the properties of enteric coatings and in comparing one type of coating with another were presented.

4. The concentration levels of talc and enteric polymer were both shown to be very important in determining the disintegration characteristics of three enteric coatings.

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# Enteric Coatings IV*

## *In Vivo* Testing of Granules and Tablets Coated With Styrene-Maleic Acid Copolymer

By JOHN G. WAGNER, WILLIAM VELDKAMP, and STUART LONG

**Preliminary tests in human subjects have indicated that the period of time which tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc resist the stomach contents of human subjects appears to be well predicted by an *in vitro* test in simulated gastric fluid U. S. P. Both freshly coated granules and stability samples of tablets coated with this formulation disintegrated rapidly in the small intestine of the dogs and human subjects, respectively. The site of disintegration was the duodenum or jejunum where optimal absorption of many therapeutic agents would be expected.**

In 1933, Lozinski and Diver (1) introduced the use of roentgenoscopy with barium sulfate tablets and the barium meal to follow the path of enteric coated tablets through the stomach and into the intestine. A year later Bukey and Brew (2) introduced reontgenography to follow the path of enteric coated dosage forms through the gastrointestinal tract of human subjects. Roentgenoscopy and roentgenography have become accepted as classicl methods to study enteric coated dosage forms and many investigators have reported such studies during the past quarter century.

One of the difficulties with the methods was pointed out by Garland (3) who noted that there could be a great difference between disintegration and absorption depending on what part of the intestine the dosage form had reached when it disintegrated. It is evident that many drugs are absorbed faster and more efficiently in the proximal parts of the small intestine. Convreur, *et al.* (4), stated that an excellent enteric coating should logically be disintegrated in the first part of the small intestine in order to allow optimum absorption of the mass medication. Hence, an enteric coated dosage form which protects the drug in the stomach but releases the drug rapidly once the dosage form enters the small intestine would be optimal for these drugs.

Some enteric coated tablets and capsules do not disintegrate very rapidly in the small intestine while others have been reported to disintegrate quite rapidly. We have calculated from the data of Crane and Wruble (5) that 174 tablets freshly coated with ammoniated shellac were emptied from the stomachs of human subjects after an average time of 3.61 hours (standard deviation 1.47 hours) and disintegrated in the small intestine after an average time of 2.55 hours (standard deviation 1.08 hours). One lot of tablets coated with ammoniated shellac was tested initially and after storage for one year at room temperature. None of the freshly coated tablets remained intact in the small intestine whereas 7 out of 35, or 20 per cent, of the one-year stability sample did remain intact in the small intestine. Similarly, Tarnowski (6) reported that shellac coated PAS granules progressively change in *in vitro* and *in vivo* disintegration behavior releasing drug much slower as they age at room temperature, and Hinkle (7) reported that radiologists observe many intact enteric coated dosage forms in the distal parts of the intestinal tract. The only conclusion with respect to intestinal disintegration time that can be drawn from the studies of Hodge, Forsyth, and Ramsey (8) is that most of the tablets and capsules coated with cellulose acetate phthalate, which they tested, apparently disintegrated from within a few minutes to up to four hours after the coated dosage forms left the stomach and entered the intestines of human patients. Convreur, *et al.* (4), reported that in five human subjects tablets containing barium sulfate and radioactive iodide and coated with cellulose acetate phthalate-diethyl phthalate emptied from the stomach after an average time of 2.63 hours (range 1.5 to 4.3 hours) and disintegrated in the intestines within ten to twenty minutes after passing through the pylorus. The same authors reported results obtained in two other patients in whom different tablet cores but with the same coating were tested. They reported that in these patients the resistance of the coating was too high and that the tablets were probably in the cecum or descending end of the sigmoid before the coating ruptured. Other investigators (9, 10) pointed out that certain enteric coatings

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The authors wish to express their gratitude to Dr. J. C. Volderauer who exposed and interpreted the roentgenograms in the clinical trials and to Dr. J. P. Webb who arranged the clinical testing.

containing cellulose acetate phthalate allow release of the medicament in the stomach and will not pass the accepted test for enteric coatings particularly after aging.

After testing many of the known enteric coatings we became acutely aware that they all suffered from basic deficiencies. The previous communication (11) described an improved enteric coating prepared from styrene-maleic acid copolymer, dibutyl phthalate, and talc. Preliminary *in vivo* testing of granules and tablets coated with this formulation by the roentgenographic method are described in this report.

## EXPERIMENTAL

**Enteric Coated Tablets.**—In the clinical studies with human subjects three different stability samples of enteric coated barium sulfate tablets were tested. The composition of these lots, CI-3, CI-6, and BI-1, have been described previously (11). Two of the lots, CI-3 and CI-6, were stored for five months at room temperature and one lot, BI-1, was stored for twelve months at 40° then for four months at room temperature before administration to the subjects.

**Protective Coated Granules.**—Pill starters containing 50% by weight of barium sulfate were prepared by applying a mixture of barium sulfate U. S. P., talc, starch, and cane sugar to sugar crystals in a pill tub using a water spray. In both simulated gastric fluid U. S. P., pH 1.2, and simulated intestinal fluid U. S. P., pH 7.5, the unprotected pill starters were completely disintegrated to a fine powder within one minute. The protective coating solution was prepared from 127.5 Gm. of styrene-maleic acid copolymer, 15.3 Gm. of di-*n*-butyl phthalate, 850 ml. of S. D. alcohol 3A, anhydrous, and 25.5 ml. of mineral oil U. S. P. In the preparation of Lot 1 an amount of 566.5 ml. of the above solution was applied in 22 aliquots to 2,300 Gm. (approx. 1.75 million granules) of the pill starters using 350 Gm. of a mixture of 80 parts by weight U. S. P. supreme talc and 20 parts by weight of magnesium stearate as a dusting powder. One-half of these coated granules were coated further with 309 ml. of the above solution, applied in 23 aliquots, using 150 Gm. of the same powder mixture as a dusting powder to produce Lot 2. Each lot of coated granules was dried for seventy-two hours at 105° F. The physical properties of the coated granules are shown in Table I.

TABLE I.—PHYSICAL PROPERTIES OF COATED GRANULES

| | Lot 1 | Lot 2 |
|---|---|---|
| Average diameter, mils | 44.0 | 46.3 |
| Average thickness of protective coating, mils | 1.6 | 2.75 |
| Average weight of protective coating, mg./granule | 0.25 | 0.44 |
| Average weight of SY-MA copolymer applied, mg./granule | 0.047 | 0.099 |

Since quantitative determination of the barium sulfate liberated from such a coated product would present great experimental difficulties disintegration tests carried out on these granules in various buffers utilized only a visual end point. In each test one hundred granules were enclosed in a glass tube which was fitted with a No. 20 mesh screen at each end. The tube was suspended from the arm of the U. S. P. disintegration apparatus and allowed to move through 100 ml. of buffer at 37°. Results obtained on Lot 2 are summarized in Table II.

**Clinical Testing of Enteric Coated Barium Sulfate Tablets by X-ray Techniques.**—Three adult, healthy male human subjects were administered two or six of the enteric coated barium sulfate tablets with a glassful of water. In all tests except one (J. G. W. with lot BI-1) the subjects ate a normal lunch, had a cup of coffee at 3:00 p. m., swallowed the tablets followed by a glassful of water at 5:00 p. m., then ate a light but normal supper at 6:00 p. m. When J. G. W. was administered lot BI-1 the subject ate a normal lunch at 12:30 p. m. and had a cup of coffee at 3:00 p. m. Two tablets were taken at 5:00 p. m., two tablets at 6:00 p. m., and two tablets at 6:30 p. m., in each case with a half glassful of water. In each test the first X-ray film was exposed at 7:00 p. m. ± five minutes. The tablets were then followed thereafter by means of the fluoroscope and X-ray films. The details of the food eaten by the subjects and the interpretations of the individual films made by Dr. J. C. Volderauer, the radiologist, are too lengthy to be reported in detail here. Sixteen of the tablets remained in the stomachs of the subjects during the observation period. Table III summarizes results obtained with these tablets. Twelve of the tablets emptied from the stomach and disintegrated in the small intestine during the observation period. Table IV summarizes the results obtained with the latter tablets. It is of interest to note that when tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc disintegrate in the small intestine they disintegrate very rapidly and completely, similar to their *in vitro* behavior in simulated intestinal fluid, pH 6.9.

**Testing the Protective Coated Granules in the Dog by X-Ray Techniques.**—The first two experiments tested the disintegration rate of the unprotected pill starters in the stomachs of dogs. A pink No. 3 capsule containing approximately 300 unprotected pill starters was administered to a dog which had been starved for twenty-four hours. A roentgenogram taken one and three-quarters of a minute later disclosed the intact capsule in the stomach of the dog. A roentgenogram taken five minutes after the first one disclosed that the capsule had broken and that all of the granules had disintegrated. A third roentgenogram taken five minutes later showed the barium sulfate well dispersed.

A pink No. 3 capsule containing approximately 310 unprotected pill starters was administered later the same day to the same dog. A roentgenogram taken one minute later showed the gelatin capsule in the process of breaking. A roentgenogram taken two and one half minutes after administration showed that the capsule had broken and that the granules had disintegrated. Additional plates taken four

TABLE II.—DISINTEGRATION TESTS ON PROTECTIVE COATED GRANULES, LOT 2

| pH of Buffer | Buffer Ingredients | Time | Observations |
|---|---|---|---|
| 1.2 | HCl-NaCl-pepsin | Up to 5 hr. | No sediment, all granules still intact on screen |
| | | 6 hr. | 95% of granules still intact and quite spherical but soft |
| 2.7 | HCl-NaCl-pepsin | 0.5 hr. | Fluid turbid; some sediment |
| | | 1 hr. | About 75% of granules intact |
| | | 2 hr. | About 50% of granules disintegrated |
| | | 3.25 hr. | Most granules remaining had cracked coatings |
| | | 5 hr. | About 75% of granules disintegrated |
| 3.2 | HCl-Potassium acid phthalate | 9 min. | Slight residue but most granules intact |
| | | 12 min. | Most granules disintegrated |
| | | 16 min. | Only fine powder remaining at bottom of beaker, all granules disintegrated and through the screen |
| 4.0 | Potassium acid phthalate | 9 min. | All granules disintegrated and through the screen |
| 7.0 | $KH_2PO_4$-NaOH | 1 min. | Fluid cloudy |
| | | 5 min. | About 95% of granules disintegrated |
| | | 7 min. | All granules disintegrated and through the screen |

TABLE III.—COMPARISON OF RESULTS OBSERVED IN MAN, WHERE TABLETS REMAINED IN THE STOMACH DURING THE OBSERVATION PERIOD, TO *In Vitro* TEST IN SIMULATED GASTRIC FLUID

| | | *In Vitro* Results in Man | | | | |
|---|---|---|---|---|---|---|
| Tablet Lot | Subject | No. of Tablets | Time Film Showed Intact Coatings, min. | Time Film Showed Coating Broken, Min. | *In Vitro* Test Av. Time ± S.E.[a] min. | Comparison Av. *in Vitro* Time / Av. *in Vitro* Time |
| CI-6. Stored five months at room temp. | D. H. G. | 1 | 239 | 327 | | |
| | C. A. S. | 2 | 240 | 329 | | |
| | J. G. W. | 1 | 181 | 241 | | |
| | J. G. W. | 1 | 241 | 311 | | |
| | | | | Av. 307 | 270 ± 11 | $\frac{307}{270} = \frac{1.1}{1}$ |
| CI-3. Stored five months at room temp. | D. H. G. | 1 | 121 | 180 | | |
| | C. A. S. | 2 | 120 | 178 | | |
| | C. A. S. | 2 | 210 | 240 | | |
| | | | | Av. 203 | 164 ± 4.7 | $\frac{203}{164} = \frac{1.2}{1}$ |
| BI-1. Stored twelve months at 40°, then four months at room temp. | D. H. G. | 2 | 300 | Not determined | Unaffected after 7.5 hr. | >300 / >450 |
| | C. A. S. | 4 | 300 | | | |

[a] Average ± standard error to coating broken end point in simulated gastric fluid U. S. P. (pH 1.2). The coating broken end point is defined as that end point where the coating has broken sufficiently so that disintegration or solution of the tablet itself has begun.

and one-quarter, five and three-quarters, and seven and one-half minutes after administration showed the barium sulfate well dispersed in the stomach as a powder.

The third experiment tested the resistance of the protective coated granules, lot 2, to the stomach contents of the dog, and the fourth experiment disclosed the site and rate of disintegration of the granules in the duodenum and small intestine. In the third experiment a No. 1 gelatin capsule containing about 325 protective coated granules, lot 2, was administered to a dog which had been starved for about seventeen hours. The amount of barium sulfate administered in this case was about 0.214 Gm. or 0.67% of the usual 30-Gm. dose given for gastrointestinal tract visualization. Films taken at intervals of ten, thirty, forty-five, sixty, seventy-five, ninety, and one hundred and five minutes after administration revealed the granules clustered in the stomach. A film taken two hours after administration had a much less intense spot from the granules in the stomach indicating that many of the granules had emptied from the stomach. A film taken fifteen minutes later indicated that all the granules had emptied from the stomach.

In the fourth experiment a No. 11 veterinary capsule containing approximately 6,940 granules of lot 2 was administered to a dog which had been starved for sixteen and one-half hours. Results of the experiment are shown in Table V.

## DISCUSSION

The time which tablets enteric coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc resist the stomach contents of human subjects appeared to be well predicted by the *in vitro* test in simulated gastric fluid, pH 1.2. Similarly, granules coated with the same agents were resistant

TABLE IV.—COMPARISON OF RESULTS OBTAINED IN MAN, WHERE TABLETS ENTERED THE SMALL INTESTINE DURING THE OBSERVATION PERIOD, TO *In Vitro* TEST IN ARTIFICIAL INESTINAL FLUID, pH 6.9

| Tablet Lot | Subject | No. of Tablets | Time from Administration, min. | Interpretation of Roentgenogram | *In Vitro* Test Average ± S. E., min.[a] |
|---|---|---|---|---|---|
| CI-6. After storage for five months at room temp. | D. H. G. | 1 | 120 | Tablets intact in lower jejunum or upper ileum | 30 ± 0.7 |
| | | | 147 and subsequent films | No trace of tablets, assumed to be completely disintegrated | |
| CI-3. After storage for five months at room temp. | J. G. W. | 4 | 123 | No trace of tablets, all four tablets completely disintegrated | 16 ± 0.3 |
| | D. H. G. | 1 | 121 and subsequent films | No trace of tablets, completely disintegrated | |
| BI-1. After storage for twelve months at 40°, then for four months at room temp. | J. G. W. | 6 | 30[b] | One tablet in first part of duodenum and five tablets in about middle of jejunum | 31 ± 1.6 |
| | | | 30 to 45 | Fluoroscopic examination indicated no change | |
| | | | 45 | One tablet still in duodenum, five tablets in jejunum and at least two of these tablets had an uneven surface indicative they had started to disintegrate | |
| | | | 54 | One tablet had moved from the duodenum to first part of the jejunum and showed signs of disintegrating; of the other five tablets, one was disintegrated into a number of fragments and the other four showed earlier signs of disintegration | |
| | | | 63 | Fluoroscopic examination indicated little change | |
| | | | 65 | One tablet in upper jejunum showed more evidence of disintegration since the last film; of the other five tablets one was completely disintegrated | |
| | | | 80 | One tablet in many fragments and fragments lower in jejunum than in previous film; other five tablets had moved further down in small bowel and definitely disintegrated | |
| | | | 90 | All tablets completely disintegrated and homogenous bolus of barium sulfate visible | |

[a] Average ± the standard error of the average disintegration time in artificial intestinal fluid, pH 6.9. The end point was taken when 99 to 100% of the entire tablet was through the screen of the U. S. P. disintegration apparatus.
[b] Thirty minutes after administration of the last two of six tablets. Two other tablets had been administered sixty minutes before this film and the remaining two tablets ninety minutes before this film.

to stomach contents of the dog for at least eight hours and were about 95% intact after six hours in simulated gastric fluid. The correlation observed with the enteric coated tablets in the dogs (12) was not nearly as good. In the dogs the *in vivo* resistance to stomach contents was considerably greater than the *in vitro* resistance to simulated gastric fluid.

All of the enteric coated tablets tested which emptied from the stomachs of the human subjects during the observation period disintegrated completely high up in the small bowel. Even the lot which had been stored for twelve months at 40° followed by four months at room temperature disintegrated completely in the upper small bowel. These tablets were attacked in the jejunum, and although no direct correlation could be made, the disintegration time in artificial intestinal fluid, pH 6.9, appeared to be a good indication of the disintegration time *in vivo*, measured from the time the tablets emptied from the stomach. In interpreting Table IV one must remember the stomach retention time of the tablets is also involved. For example, in the test of lot BI-1 in subject J. G. W. one tablet disintegrated completely twenty-four minutes after it was first observed in the small intestine and the other five tablets completely disintegrated fifty minutes after they were first observed in the small intestine.

The phenomenon of slow stomach emptying of

TABLE V.—INTERPRETATION OF ROENTGENOGRAMS OBTAINED IN THE FOURTH EXPERIMENT WITH THE DOG

| Time from Administration, min. | Interpretation of Roentgenogram |
|---|---|
| 1, 55 | Intact capsule just entered the stomach |
| 14, 8 | Capsule broken; coated granules clustered in stomach |
| 30, 1 | Granules dispersed in the stomach |
| 45, 60, 75, 90, 105, 120 | No apparent change from the above |
| 135 | Barium sulfate appeared in first loop of duodenum |
| 150 | Barium sulfate further down duodenum but less intense in first part of duodenum indicative that disintegration of some granules had occurred since the last film |
| 165 | Barium sulfate very faint in duodenum. Spot in stomach less intense than formerly |
| 180, 210 | Barium sulfate spreading throughout small bowel |
| 240 | More granules in duodenum |
| 270 | Barium sulfate dispersed and less intense in duodenum indicating disintegration had occurred since last film |
| 300 | Barium sulfate well distributed throughout bowel. Large cluster of granules still in the stomach |
| 330 | Small streak of barium sulfate again seen in duodenum |
| 420 | More barium sulfate in duodenum compared with last film taken at 390 minutes. Stomach spot less intense |
| 480 | Barium sulfate dispersed throughout small bowel |
| Test terminated | Cluster of coated granules still in the stomach but becoming much smaller |

coated granules which we observed in the dog is not necessarily restricted to granules nor to dogs. It was reported about sixty years ago that stomach emptying of human beings is a first order process (13). The rate constants or half-times for the process appear to depend upon the nature of the material introduced into the stomach and in some cases it is several hours before a given test meal is emptied. This is evidence that possibly one of the factors involved in the overall sustained effect achieved with certain coated granule preparations is the stomach-emptying rate. The slow stomach-emptying of our coated granules and rapid disintegration of the small clusters as they are released into the duodenum indicate there may be even another possible mechanism of attaining sustained action of a drug. We are merely presenting this as a hypothesis. Our other publications (14, 15) show that sustained plasma 17-hydroxycorticosteroid levels were achieved in human subjects who were administered granules containing prednisolone and coated with the entire formulation discussed in this paper.

## SUMMARY AND CONCLUSIONS

Preliminary testing of granules and stability samples of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc have indicated the following:

1. The period of time which the coated tablets resist the stomach contents of human subjects appears to be well predicted by an *in vitro* test in simulated gastric fluid, pH 1.2.

2. The coated granules resisted the stomach contents of the starved dog for at least an eight-hour period and the granules were progressively emptied from the stomach of the dog over a period of at least eight hours.

3. All of the enteric coated granules which emptied from the stomach of the dog and all of the enteric coated tablets which emptied from the stomachs of the human subjects during the observation periods disintegrated completely in the proximal part of the small bowel. This was true even for one lot of coated tablets which had been stored for twelve months at 40° and then for four months at room temperature.

4. The hypothesis is presented that slow stomach emptying of small coated granules followed by fairly rapid dissolution of small clusters of the granules as they enter the upper small intestine may be an effective method of attaining prolonged action of certain therapeutic agents

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# Enteric Coatings V*

## pH Dependence and Stability

By JOHN G. WAGNER, GEORGE W. RYAN, ERNEST KUBIAK, and STUART LONG

**Evidence is presented that the terms "stable in acid solutions" and "soluble in alkaline solutions" should not be applied to enteric coatings. The enteric substances studied dissolve in buffers of pH less than 7 and tablets coated with these substances disintegrate in fluids having a pH less than 7. It was demonstrated that the critical pH range in which tablets coated with styrene-maleic acid copolymer show a progressive decrease in disintegration time corresponds to a narrow pH range on either side of the $pK_1'$ of the copolymer. It is in this region that the greatest change in per cent ionization of the copolymer occurs. Relating these *in vitro* data with *in vivo* data, formerly reported, will require adjustment of current concepts of enteric coatings. It was shown that the disintegration times of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc in artificial intestinal fluid, pH 6.9, changed very little after storage of the coated tablets for prolonged periods at 4°, room temperature, 40°, and 47°. Tablets coated with the other enteric compositions exhibited either decreased resistance to simulated gastric fluid or markedly increased disintegration times in artificial intestinal fluids after prolonged storage. It was demonstrated that the average disintegration time observed after storage of enteric coated tablets for one month at 47° yields an excellent prediction of the average disintegration time of the tablets after storage for two years at room temperature.**

THIS COMMUNICATION has two principal purposes. First, it will be shown that the new enteric coating, formerly discussed (1), disintegrates *in vitro* at pH values very much lower than reported for any other coating. Secondly, the properties of this coating will be compared to those of other enteric coatings, both initially and after aging under various conditions.

The concept that an enteric coating is one which is "stable in acid solutions" but "soluble in alkaline solutions" should be completely forgotten. The basic definition of an enteric coating should only be in terms of resistance to stomach contents and disintegration in intestinal contents. *In vitro* tests are essential for quality control, research, and stability testing. However, we do not believe that *in vivo* results can be predicted from *in vitro* tests unless a valid correlation is established first.

During the past quarter century the *in vitro-in vivo* correlations pertaining to enteric coatings have led to a trend to find coatings which disintegrate at more and more acidic pH values. Wruble (2) reported that below pH 6.4, ammoniated shellac coatings were quite resistant but at or above this pH the coatings showed definite signs of attack. Malm, *et al.* (3), showed that a cellulose acetate phthalate, containing 10 per cent carboxyl, dissolved at pH 5.7 to 5.9 under their test conditions. Wagner, *et al.* (4), showed that a starch acetate phthalate, containing 9.2 per cent carboxyl dissolved at pH 5.55 under the same test conditions. In this paper evidence is presented that a coating, containing styrene-maleic acid copolymer, disintegrates *in vitro* at a pH as low as 3.7. It was formerly shown (5), however, that tablets and granules bearing such a coating possessed good enteric properties in the dog and man. The advantage of susceptibility to a lower (more acidic) pH is that disintegration of the coated dosage forms will occur in the proximal parts of the intestinal tract where maximal absorption of many drugs and foodstuffs occur.

Few published studies of enteric coatings have included data concerning the stability of the coatings. Scoville (6), Bukey and Rhodes (7), and Cooper (8) discussed the stability of formalized-gelatin coatings. Sampson, *et al.* (9), reported that one lot of enteric coated quinidine sulfate tablets gave imperfect dissolution after six months storage, while another lot, after similar storage, gave results essentially the same as those obtained with freshly prepared tablets. Four weeks' storage at room temperature and 0° storage were utilized by Bauer and Geraughty (10) and Stoklosa and Ohmart (11) to evaluate their enteric coatings. A patent (12) discussed the use of low pH powders with cellulose acetate phthalate to improve the stability of the coatings. In this communication the stability of many different enteric coatings after prolonged periods of storage at 4°, room temperature, 40°, and 47° is reported.

The criteria, by which the stability of the coatings were measured, were resistance to

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simulated gastric fluid U. S. P. and disintegration time in artificial intestinal fluids, pH 6.9 and 7.5.

## EXPERIMENTAL

**Materials.**—The styrene-maleic acid copolymer, starch acetate phthalate, cellulose acetate phthalate, dibutyl phthalate, and talc have been described previously (1, 4). Sorbitan monooleate used was Span 80 (Atlas Powder Co.).

Resin SC-2 was obtained from Monsanto Chemical Co. This resin contains styrene and maleic anhydride residues but is not a straight styrene-maleic acid copolymer. The resin was hydrolyzed by the method of Ferry, *et al.* (13). The hydrolyzed Resin SC-2 was dried for three days at 101°F. and 10% relative humidity.

The compositions of the various artificial gastric and intestinal fluids used in this study are shown in Table I.

**Enteric Coated Tablets.**—Lots BI-1, BI-2, BII-1, BII-2, CII-4, CII-5, and CII-6, coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc, have been described previously (1). Lot N was coated with the same mixture of ingredients. Lots D through I were coated with ammoniated shellac as described in the patent of Wruble (14). Lot T was comprised of tablets of lot BII-1 which were film-coated with carboxymethylcellulose and talc after storing for one hundred and fifty-five days at room temperature. Lot U was comprised of tablets of lot BI-2 which were film-coated with carboxymethylcellulose and talc after storing for one hundred fifty-seven days at room temperature. The compositions of the remainder of the enteric coatings are shown in Table II. All tablets were coated by conventional pan-coating methods using talc as a dusting powder.

## METHODS

**Potentiometric Titrations.**—About 0.3 Gm., accurately weighed, of styrene-maleic acid copolymer or hydrolyzed Resin SC-2 was suspended in 200 ml. of boiling water. The suspension was heated on the steam bath with stirring until a homogeneous solution was obtained. The solution was cooled to room temperature and then titrated with 0.1 *N* sodium hydroxide. The pH of the solution after the addition of each increment of standard base was determined with a Cambridge pH meter fitted with an electric eye. The pH meter was calibrated with Harleco buffer, pH 7.00, at the temperature of the test solution. The inflection point was determined by plotting $\Delta$ pH/$\Delta V$ against $V$, where $V$ was the volume of standard base added.

**Disintegration Tests.**—In all tests, the U. S. P. disintegration apparatus, without plastic disks, was used. The fluids were maintained at 37 ± 2°. Unless otherwise indicated the end point for disintegration tests in artificial intestinal fluids was taken when 99 to 100% of the entire tablet was

TABLE I.—COMPOSITION OF FLUIDS USED IN THE DISINTEGRATION TESTS

| Ingredient | Artificial Gastric Fluids pH 1.2 | Artificial Gastric Fluids pH 3.0 | Artificial Intestinal Fluids pH 3.7 | Artificial Intestinal Fluids pH 4.0 | Artificial Intestinal Fluids pH 4.8 | Artificial Intestinal Fluids pH 6.9 | Artificial Intestinal Fluids pH 7.5 |
|---|---|---|---|---|---|---|---|
| Pancreatin 3× U. S. P. | [a] | ... | 3.33 Gm. | 3.33 Gm. | 3.33 Gm. | 3.33 Gm. | [b] |
| Ox bile extract U. S. P. | [a] | ... | 4.0 Gm. | 4.0 Gm. | 4.0 Gm. | 4.0 Gm. | [b] |
| Pepsin | [a] | 3.2 Gm. | ... | ... | ... | ... | [b] |
| 0.2 *M* Potassium biphosphate | [a] | ... | ... | ... | ... | 250.0 ml. | [b] |
| 0.2 *M* Potassium biphthalate | [a] | ... | 250.0 ml. | 250.0 ml. | 250.0 ml. | ... | [b] |
| 0.1 *N* Hydrochloric acid | [a] | 204.0 ml. | 49.75 ml. | ... | ... | ... | [b] |
| 0.2 *M* Sodium hydroxide | [a] | ... | ... | 2.0 ml. | 118.25 ml. | 140.0 ml. | [b] |
| Deionized water, *q. s.* | [a] | 1,000 ml. | 1,000 ml. | 1,000 ml. | 1,000 ml. | 1,000 ml. | [b] |

[a] Simulated gastric fluid U. S. P. [b] Simulated intestinal fluid U. S. P.

TABLE II.—COMPOSITION OF ENTERIC COATINGS

| Lot of Enteric Coated Tablets | Subcoated Tablets Used | Amount of Ingredient Applied Per Tablet: Styrene-Maleic Acid Copolymer Type I, mg. | Hydrolyzed Resin SC-2 mg. | Starch Acetate Phthalate, mg. | Cellulose Acetate Phthalate, mg. | Shellac, mg. | Mineral Oil, ml. | Dibutyl Phthalate, mg. | Sorbitan Monooleate, ml. | Propylene Glycol U. S. P., ml. | Talc, mg. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| J | I[a] | 25 | ... | .. | ... | 3 | ... | ... | ... | ... | 136 |
| K | I | 25 | ... | .. | ... | 3 | ... | 3 | ... | ... | 125 |
| L | IV[a] | ... | ... | 21 | ... | .. | 0.0063 | ... | 0.0021 | 0.0063 | 79 |
| M | [d] | ... | ... | 15 | ... | .. | ... | 1.8 | ... | ... | 42 |
| N | [e] | 37.5 | ... | .. | ... | .. | ... | 4.5 | ... | ... | 234 |
| O | [f] | ... | 17.3 | .. | ... | .. | ... | 3.5 | ... | ... | 57 |
| P | [e] | ... | 22.5 | .. | ... | .. | ... | 4.5 | ... | ... | 142 |
| Q | I | 7.5 | ... | .. | 7.5 | .. | 0.005 | 3 | ... | ... | 80 |
| R[b] | I | 25 | ... | .. | 12 | .. | 0.0036 | 3 | 0.0012 | 0.0036 | 122 |
| S[c] | I | 40 | ... | .. | 12 | .. | 0.0063 | 4.8 | 0.0012 | 0.0036 | 198 |

[a] See Table II of previous communication (15).
[b] A double enteric coated tablet with internal SY-MA copolymer coating and external cellulose acetate phthalate coating separated by a carboxymethylcellulose barium sulfate layer.
[c] A double enteric coated tablet with internal cellulose acetate phthalate coating and external SY-MA copolymer coating separated by a carboxymethylcellulose-barium sulfate layer.
[d] Same subcoated tablet as used for C-I, C-II, and C-III series [see Table I of reference (1)].
[e] A subcoated tablet with average weight of 0.8 Gm. per tablet.
[f] A subcoated ammonium chloride tablet with average weight of 0.766 Gm. per tablet.

through the screen of the U. S. P. disintegration apparatus. In the case of tablets coated with ammoniated shellac, the end point was not nearly as sharp since frequently the coatings formed a sack through which the core tablet contents were leached by the fluids.

Two studies were carried out. The first study consisted of two parts: (*a*) a study of the dependence of acid resistance and disintegration time of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc on the pH of the test fluid; and (*b*) a study of the dependence of the disintegration time of tablets coated with ammoniated shellac on the pH of the test fluid. The second study was concerned with the stability of enteric coatings with respect to their disintegration behavior. Tablets with different enteric coatings were tested initially and after storage for periods of time from one to twenty-four months at 4°, room temperature, and at two elevated temperatures, 40 and 47°. In many of these tests the disintegration time was determined independently by two different laboratories, designated "A" and "B" in Tables VI and VII. In the range from fifteen to fifty minutes, in which most of the average disintegration times of the enteric coated tablets fall, the agreement between the two laboratories was excellent.

## RESULTS

**Potentiometric Titrations.**—The styrene-maleic acid copolymer was found to have an apparent $pK_1'$ of 4.24. This was an average of five determinations which ranged from 4.20 to 4.27. Garrett and Guile (16) reported a $pK_1'$ of 4.25 for the copolymer. The hydrolyzed Resin SC-2 was found to have an apparent $pK_1'$ of 4.83. This was an average of four determinations which ranged from 4.70 to 4.94.

Accurate $pK_1'$ values for starch acetate phthalate, cellulose acetate phthalate, and shellac could not be obtained in water or aqueous-organic solvent mixtures due to the tendency of the polymers to precipitate during the titrations.

**Dependence of Acid Resistance and Disintegration Time on the pH of the Test Fluid.**—Results obtained with three lots of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc are shown in Table III.

Within experimental error, two of the lots, C II-4 and C II-6, exhibited the same resistance to both artificial gastric fluids, pH 1.2 and pH 3.0. In artificial intestinal fluids, having pH values of 3.7, 4.0, and 4.8, the disintegration time of lot C II-5 decreased as the pH increased. However, the disintegration time reached an apparent minimum at pH 4.8 because the disintegration times at pH 6.9 and 4.8 were not significantly different.

Starch acetate phthalate coatings are much less sensitive to low pH fluids than the styrene-maleic acid copolymer coating. For example, in the artificial intestinal fluid, pH 4.8, lot C III-2 exhibited only broken coatings after two hundred and thirty minutes, whereas, as formerly reported (1) these tablets were completely disintegrated after an average time of forty-one minutes in artificial intestinal fluid, pH 6.9.

Tablets coated with ammoniated shellac require media of even higher pH in order to obtain reasonably low disintegration times. Table IV is a tabulation of disintegration times of fourteen stability samples of six different lots of tablets coated with ammoniated shellac.

The results indicate that the shellac-coated tablets disintegrate much more rapidly in simulated intestinal fluid, pH 7.5, than in artificial intestinal fluid, pH 6.9.

**Stability of Enteric Coatings With Respect to Their Disintegration Behavior.**—Results shown in Tables IV and V indicate that tablets enteric coated with shellac exhibit progressively increasing average disintegration times with increasing storage time at room temperature and at elevated temperatures.

In contrast to styrene-maleic acid copolymer alone, shellac in combination with the copolymer (lots J and K) causes a marked increase in disintegration time after storage of the tablets for one month at 47°.

The disintegration times of tablets coated with starch acetate phthalate also increase markedly with increase in storage time at room temperature and at elevated temperatures. The disintegration times increase more rapidly with age when Span 80 and propylene glycol are used with starch acetate phthalate than when dibutyl phthalate is used as a plasticizer. This difference can be seen by comparing lots L and M in Table V.

Tablets coated with a mixture of cellulose acetate phthalate and styrene-maleic acid copolymer (lot Q, Tables VI and VII) became more sensitive to simulated gastric juice after aging at room temperature and elevated temperatures.

Tablets coated with styrene-maleic acid co-

TABLE III.—INITIAL DISINTEGRATION TESTS ON TABLETS COATED WITH STYRENE-MALEIC ACID COPOLYMER, DIBUTYL PHTHALATE, AND TALC IN VARIOUS SYNTHETIC GASTRIC AND INTESTINAL FLUIDS

| Test Fluid | Tablet Lot C II-4 Av. | C II-4 S. E.[a] | C II-5 Av. | C II-5 S. E. | C II-6 Av. | C II-6 S. E. |
|---|---|---|---|---|---|---|
| Simulated gastric fluid, U. S. P., pH 1.2 | 165[b] [c] | 8.2 | 193[b] | 8.6 | 273[b] [c] | 12.1 |
| Artificial gastric fluid, pH 3.0 | 180[b] [c] | 6.6 | ... | ... | 268[b] [c] | 19.7 |
| Artificial intestinal fluid, pH 3.7 | ... | ... | 49.5[d] | 2.5 | ... | ... |
| Artificial intestinal fluid, pH 4.0 | ... | ... | 41.6[d] | 1.5 | ... | ... |
| Artificial intestinal fluid, pH 4.8 | ... | ... | 24.3[d] [c] | 0.67 | ... | ... |
| Artificial intestinal fluid, pH 6.9 | ... | ... | 23.8[d] [c] | 0.23 | ... | ... |

[a] Standard error.
[b] Average time (in minutes) to coating attacked end point as defined previously (15).
[c] There are no significant differences in the pairs of averages which are bracketed.
[d] Average time (in minutes) for complete disintegration of the coated tablet.

TABLE IV.—AVERAGE DISINTEGRATION TIMES[a] AND THEIR STANDARD ERRORS OF TABLETS ENTERIC COATED WITH AMMONIATED SHELLAC[b]

| Lot | Storage Conditions of Tablets Before Test — Temperature | Time, Months | Artificial Intestinal Fluid, pH 6.9 — Av. | S. E. | Simulated Intestinal Fluid, pH 7.5 — Av. | S. E. |
|---|---|---|---|---|---|---|
| D | RT[c] | 6 | 83 | 4.2 | 37 | 1.8 |
| D | 40° | 3 | 136 | 4.4 | 78 | 3.4 |
| E | RT | 3 | 33.7 | 1.6 | 32.5 | 1.2 |
| E | 40° | 3 | 103 | 3.8 | 58 | 2.5 |
| E | 47° | 3 | >155 | ... | >155 | ... |
| F | RT | 3 | 50.0 | 3.3 | 22.2 | 0.87 |
| F | 40° | 3 | 92 | 3.6 | 73 | 6.4 |
| F | 47° | 3 | >240 | .. | 163 | 12.5 |
| G | 40° | 3 | >240 | ... | 64 | 3.9 |
| G | 47° | 3 | >240 | | >240 | ... |
| H | RT | 3 | 52.5 | 1.7 | 32.2 | 0.87 |
| H | 40° | 3 | 132 | 7.0 | 50. | 2.7 |
| I | RT | 3 | 50.0 | 1.7 | 29.8 | 0.82 |
| I | 40° | 3 | 145 | 4.6 | 52.5 | 2.5 |

[a] Based on 6 tablets in each test fluid.
[b] Prior to the disintegration tests the tablets were unaffected by immersion in simulated gastric fluid U. S. P. for one hour.
[c] Room temperature.

TABLE V.—AVERAGE DISINTEGRATION TIMES[a] AND THEIR STANDARD ERRORS OF VARIOUS ENTERIC COATED TABLETS IN ARTIFICIAL INTESTINAL FLUID, pH 6.9

| Lot | Enteric Substance | Initial Av. | Initial S. E. | Stability Sample: 3 Months at RT Av. | 3 Months at RT S. E. | 6 Months at RT Av. | 6 Months at RT S. E. | 1 Month at 47° Av. | 1 Month at 47° S. E. | 3 Months at 47° Av. | 3 Months at 47° S. E. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| J | Styrene-maleic acid copolymer | 49.2 | 0.9 | ... | ... | ... | ... | 137.7 | 3.9 | ... | ... |
| K | plus shellac | 33.8 | 0.8 | ... | ... | ... | ... | 149.8 | 8.5 | ... | ... |
| L | Starch acetate phthalate | 58.2 | 1.5 | 65.8 | 1.7 | 88.9 | 4.9 | 177.5 | 2.1 | ... | ... |
| M | | 35.7 | 1.0 | 40.3 | 0.9 | 40.8 | 1.3 | 90 | 2.5 | 115 | 2.8 |
| A | Ammoniated shellac | 25.9 | 0.5 | 42.6 | 1.0 | 82.8 | 4.2 | 151 | 19 | >240 | ... |
| B | | 22.5 | 0.8 | 111 | 6.6 | ... | ... | 170 | 21 | >240 | ... |

[a] Based on 12 tablets.

TABLE VI.—AVERAGE DISINTEGRATION TIMES AND THEIR STANDARD ERRORS OF VARIOUS ENTERIC COATED TABLETS IN ARTIFICIAL INTESTINAL FLUID, pH 6.9[a]

| Type of Coating | Lot | | Initial Av. | Initial S. E. | Storage Time at Room Temperature, months: 3 Av. | 3 S. E. | 6 Av. | 6 S. E. | 12 Av. | 12 S. E. | 18 Av. | 18 S. E. | 24 Av. | 24 S. E. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Styrene maleic acid copolymer | BI-1 | A[b] | 15.1 | 0.31 | 16.5 | 0.55 | | | | | | | | |
| | | B[c] | 15 | | 15 | | 16.8 | 0.27 | 13.2 | 0.14 | 21.8 | 0.44 | 18.6 | 0.34 |
| | BII-1 | A | 41.0 | 0.80 | 42.5 | 0.74 | | | | | | | | |
| | | B | 40 | | 40 | | 45.0 | 0.43 | 46.0 | 0.82 | 49.8 | 0.93 | 54.3 | 0.85 |
| | BII-2 | A | 41.3 | 0.71 | 43.4 | 1.1 | | | | | | | | |
| | | B | 40 | | 40 | | 44.2 | 0.49 | 41.7 | 0.58 | 50.7 | 0.71 | 52.2 | 0.68 |
| | N | A | 46.4 | 1.6 | | | | | | | | | | |
| | | B | 45 | | 51.5 | 0.68 | 52.9 | 0.42 | 51.8 | 1.3 | 53.0 | 1.3 | 49.1 | 1.0 |
| | BI-2 | A | 46.8 | 0.84 | 51.7 | 1.4 | | | | | | | | |
| | | B | 50 | | 40 | | 52.4 | 0.91 | 56.7 | 0.48 | 59.8 | 1.0 | 65.3 | 0.67 |
| Hydrolyzed Resin SC-2 | O | A | 15.8 | 0.45 | 18.6 | 1.1 | | | | | | | | |
| | | B | 20 | | 20 | | 20.9 | 0.50 | 17.3 | 1.6 | 23.4 | 1.9 | 26.9 | 0.36 |
| | P | A | 23.8 | 0.80 | | | | | | | | | | |
| | | B | 20 | | 21.5 | 0.53 | 24.2 | 0.09 | 29.0 | 0.64 | 29.8 | 0.60 | 32.3 | 1.1 |
| Mixture of styrene-maleic acid copolymer and cellulose acetate phthalate | Q[d] | A | 17.3 | 0.55 | 20.5 | 0.68 | | | | | | | | |
| | | B | 20 | | | | 15.2 | 0.67 | 19.7 | 0.75 | 20.2 | 0.59 | 23.5 | 0.81 |
| Double enteric coated tablets (see Table II) | R | A | 52.3 | 0.93 | 53.0 | 1.2 | | | | | | | | |
| | | B | 45 | | 40 | | 41.3 | 0.61 | 41.7 | 0.89 | 44.0 | 1.0 | 44.5 | 0.93 |
| | S | A | 56.9 | 1.6 | 64.3 | 1.2 | | | | | | | | |
| | | B | 65 | | 55 | | 65.4 | 1.3 | 60.3 | 1.5 | 61.4 | 1.5 | 67.3 | 1.4 |
| Film coated over enteric coating (see Table II) | T | A | | | | | | | | | | | | |
| | | B | 44.0 | 0.95 | 47.5 | 1.0 | 57.8 | 1.0 | 56.8 | 1.4 | 61.5 | 1.1 | 60.8 | 0.77 |
| | U | A | | | | | | | | | | | | |
| | | B | 61.8 | 0.60 | 53.9 | 0.63 | 62.3 | 0.28 | 74.2 | 1.7 | 69.3 | 1.7 | 67.0 | 0.95 |

[a] Unless otherwise indicated, all tablets tested were unaffected by immersion in simulated gastric fluid for two hours prior to the disintegration test, time in minutes.
[b] Disintegration time determined by laboratory "A" based on 12 tablets.
[c] Disintegration time determined by laboratory "B" based on 12 tablets.
[d] Some of the tablets were noticeably affected by the two-hour treatment with simulated gastric fluid U. S. P.

TABLE VII.—AVERAGE DISINTEGRATION TIMES AND THEIR STANDARD ERRORS OF VARIOUS ENTERIC COATED TABLETS IN ARTIFICIAL INTESTINAL FLUID, pH 6.9[a]

| Type of Coating | Tablet Lot | Storage Conditions: 24 mo. at 4° Av. | S. E. | 1 mo. at 47° Av. | S. E. | 3 mo. at 40° Av. | S. E. | 3 mo. at 47° Av. | S. E. | 6 mo. at 40° Av. | S. E. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Styrene-maleic acid copolymer | BI-1 | 16.3[b] | 0.31 | 16.4[b] | 0.41 | 18.4[b] | 0.31 | 19.5[b] | 0.37 | 33.5[b] | 0.44 |
| | BII-1 | 46.3[b] | 0.77 | 53.3[b] | 0.60 | 57.6[c] | 1.4 | 61.1[b] | 0.77 | 45.8[c] | 0.62 |
| | BII-2 | 43.5[b] | 0.70 | 53.3[b] | 0.74 | 47.4[c] | 1.9 | 55.6[b] | 0.68 | 41.3[c] | 0.60 |
| | N | 46.6[b] | 0.66 | 50.7[b] | 2.2 | 48.4[c] | 1.5 | 47.8[c] | 0.62 | 43.7[c] | 0.88 |
| | BI-2 | 55.0[c] | 0.84 | 65.3[b] | 0.73 | 54.4[c] | 0.88 | 73.0[b] | 0.64 | 48.5[c] | 0.34 |
| Hydrolyzed Resin SC-2 | O | 17.9[c] | 0.52 | 26.4[b] | 1.1 | 36.1[b] | 1.5 | 43.0[b] | 0.86 | 27.5[c] | 0.74 |
| | P | 27.2[c] | 0.52 | 32.2[b] | 0.89 | 32.6 | 1.2 | 36.8[c] | 0.71 | 38.4[c] | 1.5 |
| Mixture of styrene maleic acid copolymer and cellulose acetate phthalate | Q | 22.0[c] | 0.42 | 20.3[b] | 0.70 | 22.8[c,d] | 0.64 | 22.0[b] | 1.1 | 24.0[c,e] | 1.3 |
| Double enteric coated tablets (see Table II) | R | 52.2[c] | 1.8 | 47.2[b] | 1.0 | 58.5[c] | 1.2 | 48.7[b] | 1.0 | 35.6[c] | 1.2 |
| | S | 63.3[c] | 1.4 | 81.1[b] | 1.8 | 104.9[c] | 3.9 | 110.3[b] | 1.9 | 117.3[c] | 2.4 |
| Film-coated over enteric coating (see Table II) | T | 54.0[c] | 0.87 | 77.2[c] | 0.82 | 60.1[c] | 1.1 | 121.2[c] | 8.0 | 127.0[c] | 3.5 |
| | U | 62.1[c] | 0.66 | 71.0[c] | 1.1 | 63.3[c] | 3.5 | 82.3[c] | 3.1 | 81.3[c] | 8.1 |

[a] Unless otherwise indicated, all tablets tested were unaffected by immersion in simulated gastric fluid for two hours prior to the disintegration test, time in minutes.
[b,c] Disintegration times determined by laboratories "A" and "B," respectively. Twelve tablets were used in each case.
[d,e] Two of twelve and nine of twelve tablets, respectively, had broken coatings after exposure to simulated gastric fluid for two hours.

polymer or with hydrolyzed Resin SC-2 exhibited only very slight increases in disintegration times after storage for prolonged periods at room temperature or at elevated temperatures. Five lots of tablets coated with styrene-maleic acid copolymer and two lots coated with hydrolyzed Resin SC-2 were tested. After storage for either two years at room temperature or one month at 47° these seven lots exhibited an average increase in disintegration time of only ten minutes in artificial intestinal fluid, pH 6.9.

The application of a film coating of sodium carboxymethylcellulose and talc over a styrene-maleic acid coating caused the disintegration times to increase upon aging of the tablets more than could be accounted for by the small increase in disintegration time due to aging of the styrene-maleic acid copolymer coating alone. This can be seen by comparing the data for lot T with that of lot BII-1 and for lot U with lot B I-2 in Tables VI and VII. The reason for this effect of the film coating is being investigated further.

None of the tablets coated with styrene-maleic acid copolymer or hydrolyzed Resin SC-2 showed any decreased acid resistance after prolonged storage at room temperature or at elevated temperatures as evidenced by a two-hour immersion in simulated gastric fluid with the standard U. S. P. apparatus. In fact, the resistance at pH 1.2 of such coated tablets usually increases with aging, particularly at elevated temperatures. For example, lot BI-1 had an initial average resistance time of four and three-quarter hours in simulated gastric fluid. After storage for twelve months at 40° followed by four months at room temperature, the tablets were unaffected by immersion in simulated gastric fluid for seven and one-half hours at 37°; after an additional thirty-seven hours in the fluid at room temperature, the tablet coatings had only small cracks and most of the core tablets were still intact.

## DISCUSSION

**Relationship Between Disintegration Rate, Apparent pK′ of Enteric Substance, and pH of Test Fluid.** —Both of the polyelectrolytes, styrene-maleic acid copolymer and hydrolyzed Resin SC-2, have $\alpha,\beta$-dicarboxylic acid units. One of the carboxyl groups of each unit is considerably more acidic than the other and is completely ionized before the other starts to dissociate. Since we are only concerned with pH values below the neutral point (pH 7), the ensuing discussion pertains only to the more acidic carboxyl of each $\alpha,\beta$-dicarboxylic acid unit.

Substituting the $pK_1'$ of 4.24 for styrene-maleic acid copolymer into the equation of Albert (17), one can calculate that approximately 99% of the carboxyl groups will be ionized at pH 6.24 but only approximately 1% will be ionized at pH 2.24. The data of Ferry, *et al.* (13), indicate that between 1 and 10% of the carboxyl groups have to be ionized before solution of the polymer occurs in the presence of dilute hydrochloric acid. When the copolymer is used as a coating agent with a plasticizer and a dusting powder, the exact pH at which the resultant coatings dissolve, or allow rapid release of the enclosed medicament, depends to some extent on the exact composition of the coating and the nature and solubility of the medicament in the core. Acid resistance of such coatings is usually good up to about pH 3 or slightly above. At about pH 3.4 and above the rate of disintegration of the coatings become quite rapid.

It would be interesting to compare the change in rate of disintegration with change in pH for different coatings. Unfortunately, we cannot readily measure the rate of disintegration of enteric coated tablets. Only the disintegration time is measured. A reasonable assumption, however, is that the disintegration rate constant, $R$, is inversely proportional to the average disintegration time, $T_i$, i. e., $R \propto 1/T_i$. Hence, if we determine values of $\Delta\left(\frac{1}{T_i}\right)\Big/\Delta$ pH, we should obtain values which are directly proportional to values of $\Delta R/\Delta$ pH. Using the data for lot C II-5 in Table III, a plot of $1/T_i$ *versus* pH, in the pH range 3.7 to 4.8, yields a straight line with slope, $\Delta\ (1/T_i)/\Delta$ pH, of 0.020. At pH 3.7, 4.0, and 4.8 the more acidic carboxyls of styrene-maleic acid copolymer are 22, 36, and 78% ionized, respectively. Hence the pH range in which the disintegration rate is increasing (or disintegration time decreasing) markedly with an increase in pH,

corresponds to the pH range where the per cent ionization of the polymer is changing most rapidly, i. e., in a narrow pH range on either side of the $pK_1'$ of the copolymer.

Nine of the lots of tablets, which were coated with ammoniated shellac, have finite and distinctly different disintegration times at pH 6.9 and 7.5 (see Table IV). The average value of $\Delta (1/T_t)/\Delta$ pH calculated for these lots was 0.020. It seems reasonable to conclude that the apparent pK′ of shellac lies in the range 6.9 to 7.5.

**Relationship Between Critical *In Vitro* pH Range and Enteric Properties.**—We may define the critical *in vitro* pH range of an enteric coating as the pH range in which the disintegration rate increases rapidly or the disintegration time decreases rapidly. The critical *in vitro* pH range for the styrene-maleic acid copolymer-dibutyl phthalate-talc coating on lot C II-5 was shown to be pH 3.7 to 4.8 and that for the ammoniated shellac-talc coatings to be about pH 6.9 to 7.5. The critical *in vitro* pH ranges for starch acetate phthalate and cellulose acetate phthalate coatings apparently lies between these two pH ranges, i. e., between 3.7–4.8 and 6.9–7.5.

Disintegration at such low pH values, as observed for the styrene-maleic acid coating, is new in the enteric field. However, we have shown previously (5, 15) that such coatings function as enteric coatings in the dog and human subjects. The results should, however, not be too surprising. Maltby (18) found that after administration of a protein meal to a group of 147 patients without history of gastric disease or pernicious anemia, 65% of the cases had gastric contents with pH values ranging from 1.5 to 2.5 and 86% had gastric contents with pH values ranging from 1.5 to 3.5. A great deal of evidence indicates that the intestinal contents range from pH 3.6 to pH 7.9. To ensure that the enteric coatings disintegrate soon after reaching the small intestine we believe that enteric coated tablets should disintegrate on the acid side at as low a pH as 3.7.

**The Stability of the Enteric Coatings Studied.**—Such very small changes in average disintegration time in artificial intestinal fluid, as observed for the tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc, have not been previously reported for any enteric coating. The few stability tests reported for other coatings have usually involved much shorter storage times and have not included elevated temperature studies (6–12). Some enteric coatings show decreased resistance to acid solutions or the coatings crack or craze after aging (11, 12). The increase in acid resistance of styrene-maleic acid copolymer coatings upon aging is a desirable property.

Other investigators (19, 20) made similar observations as ours with respect to shellac-coated tablets. We would like to point out, however, that such increases in disintegration time do not necessarily occur when shellac is used in small quantities as a water-proofing agent before application of sugar or other coatings to tablets.

**Correlation of Average Disintegration Times of Tablets After Storage for Twenty-four Months at Room Temperature With Average Disintegration Times of the Same Tablets After Storage for One Month at 47°.**—Use of the Arrhenius equation relating the rate of reaction with temperature was not practically possible with the enteric coated tablets tested because of the following reasons: (*a*) changes in disintegration time with increase in age were very small for these newer coatings, and (*b*) higher temperatures than 47° could not be used because reactions occurred in the coatings which were known not to occur at 47° or below. It was desirable, however, to establish a short term, high temperature stability test which would give a satisfactory estimate of the average disintegration time which would be observed if the enteric coated tablets were stored for two years at room temperature.

Based on the twelve lots of enteric coated tablets studied, it has been found that the average disintegration time observed after storage of tablets for one month at 47° gives an excellent prediction of the average disintegration time of the tablets after storage for twenty-four months at room temperature. Figure 1 shows such a relationship using the values of average disintegration time reported in Tables VI and VII. However, the correlation is improved if those average disintegration times observed by laboratory "A" are corrected to those which would be expected to be reported by laboratory "B". The first ten average disintegration times, $T_a$ of tablets stored for one month at 47°, as reported by laboratory "A", were transformed into the predicted disintegration times, $\hat{T}_b$, which would be expected to be reported by laboratory "B", using the equation $\hat{T}_b = 0.852\ T_a + 4.2$ which was based on 49 lots of tablets. The average disintegration times of the tablets after storage for twenty-four months at room temperature were then plotted against the average disintegration times of the tablets after storage for one month at 47°, $T_b$ or $\hat{T}_b$.

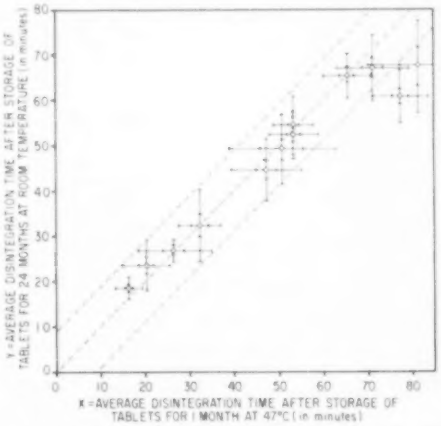


Fig. 1.—Correlation of disintegration times of twelve lots of enteric coated tablets in artificial intestinal fluid, pH 6.9, after storage for twenty-four months at room temperature with disintegration times after storage of the tablets for one month at 47°. Center dotted line represents the relation $\hat{y} = x$. Outer dotted lines represent 95% confidence limits of the center dotted line. Inner bars on solid lines represent 95% confidence limits about the average disintegration time plotted as circles. Outer bars on solid lines represent 95% confidence limits of individual tablet disintegration times.

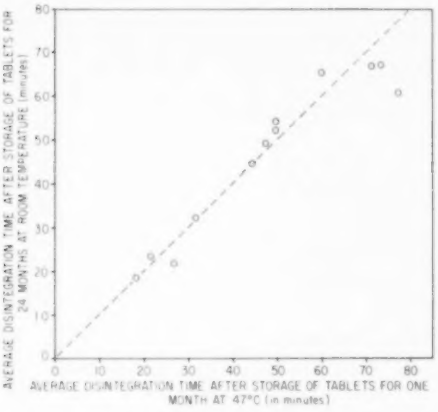


Fig. 2.—Correlation of disintegration times of twelve lots of enteric coated tablets in artificial intestinal fluid, pH 6.9, after storage for twenty-four months at room temperature with disintegration times after storage for one month at 47°. Average disintegration times reported by laboratory A have been converted to those which would be expected to be reported by laboratory B.

The latter plot is shown in Fig. 2. The "least squares" lines were shown to have a slope not differing significantly from unity. Hence, within the error of the measurements and the dispersion of the averages, the disintegration times of tablets after storage for one month at 47° is an excellent estimate of the disintegration time observed after storage for twenty-four months at room temperature.

## SUMMARY AND CONCLUSIONS

1. The resistance of tablets enteric coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc to artificial gastric fluid is not significantly different at pH 1.2 or pH 3.0. In artificial intestinal fluids the disintegration time decreases over the pH range 3.7 to 4.8 but the disintegration time at pH 4.8 is not significantly different from the disintegration time at pH 6.9.

2. The average disintegration time of tablets enteric coated with either styrene-maleic acid copolymer or hydrolyzed Resin SC-2 increases only by an average of ten minutes (range four to fifteen minutes) after storage of the tablets for two years at room temperature or for one month at 47°.

3. Tablets coated with starch acetate phthalate, ammoniated shellac, or shellac plus styrene-maleic acid copolymer exhibited markedly increased disintegration times in artificial intestinal fluid, pH 6.9, after aging at room temperature or elevated temperature.

4. None of the lots of tablets coated with styrene-maleic acid copolymer or hydrolyzed Resin SC-2 showed any decreased resistance to acid at pH 1.2 after prolonged storage at room temperature or elevated temperatures as evidenced by immersion in simulated gastric fluid for two hours. After storage at elevated temperatures for prolonged periods such coated tablets exhibit markedly increased acid resistance at pH 1.2 compared with their initial acid resistance at pH 1.2.

5. Tablets coated with a mixture of cellulose acetate phthalate and styrene-maleic acid copolymer exhibited decreased acid resistance on storage at room temperature and at elevated temperatures.

6. The average disintegration time observed after storage of enteric coated tablets for one month at 47° was shown to give an excellent prediction of the average disintegration time of the tablets after storage for two years at room temperature. The correlation was based on twelve lots of tablets and included five different types of coatings.

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# The Preparation and Antibacterial Action of Metal Chelates of Some Antitubercular Agents, Amino Acids, and Peptides*

By FREDERICK T. COUNTER, Jr., RONALD N. DUVALL, WILLIAM O. FOYE, and RAYMOND W. VANDERWYK

**A series of eighteen metal chelates and complexes of some α-amino acids, peptides, isonicotinoyl hydrazide, *p*-aminosalicylic acid, *p*-acetylaminobenzal thiosemicarbazone, and biotin were screened for antibacterial activity against ten microorganisms. Quantitative tests were carried out on six of these compounds by a modified serial tube dilution method; these compounds were the copper complex and chelate of PASA, cobalt chelates of INH and methionine, and copper chelates of glycyl-DL-alanine and biotin. Evidence indicated that the complexes of methionine and biotin were more active than the metal ions, but the action of the chelates of the antitubercular agents appeared to depend in part on dissociation of the chelates.**

THE ACTION of chelating agents against bacteria has received some attention, but the question as to the dependence of this activity on the chelating agent or the chelate itself has not been thoroughly resolved. Erlenmeyer and workers (1), for instance, found a sizable number of compounds capable of metal chelation to have antibacterial and antituberculotic activity in the presence of definite concentrations of certain metal ions. Albert (2) has investigated the antibacterial action of 8-hydroxyquinoline and provided good evidence that this agent exerts its effect only in the form of toxic metal chelates. Cymerman-Craig and Rubbo (3) have shown that isonicotinoyl hydrazide (INH) derivatives incapable of metal chelation have no antituberculotic action, and Foye and Duvall have shown both *p*-aminosalicylic acid (PASA) (4) and INH (5) to be as effective against tubercle bacilli in the form of certain metal chelates. Garattini and Leonardi (6) have also shown that among a large series of compounds having inhibitory effects against *M. tuberculosis*, the most effective were two powerful chelating agents, *o*-phenanthroline and sodium diethyldithiocarbamate, which could hardly be expected to exist in aqueous media without chelating any metal ions present.

It was therefore felt that antibacterial testing of a number of metal chelates of agents having little or no antibacterial activity might be illuminating. Although positive results from this experiment would not necessarily explain the action of individual antibacterials, they would indicate that the metal chelate structure, *per se*, is capable of inhibiting the growth of microorganisms. Among the metal chelates selected for this purpose, several antitubercular agents, which show little activity against other bacteria, were included in the hope that further information regarding the importance of chelation in their action against tubercle bacilli might be found.

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## METHODS

**Materials.**—The PASA used was a gift of Parke, Davis and Co., the INH was a gift of the Massachusetts General Hospital, and the cupric, ferric, and zinc oxides were Baker's C. P. analyzed reagents. The preparation of the copper complex and copper and iron chelates of PASA (4), and the copper, iron, zinc, and cobalt chelates of INH (5) has already been described. The preparations of the remainder of the chelates follow.

*p-Aminobenzaldehyde Thiosemicarbazone.*—This compound was synthesized by the method of Beard and Hodgson (7). A 65% yield of product was obtained which melted at 196–197° [lit. m. p. 198° (8)].

*p-Acetylaminobenzaldehyde Thiosemicarbazone (Tibione).*—The acetylation procedure of Behnisch, *et al.* (9), was used. A 52% yield of product was obobtained which melted at 228–229° [lit. m. p. 230° (9)].

*Copper Chelate of p-Acetylaminobenzaldehyde Thiosemicarbazone.*—This was prepared by the slow addition, with stirring, of 1.88 Gm. (0.0075 mole) of copper sulfate pentahydrate in 400 ml. of water to 3.54 Gm. (0.015 mole) of *p*-acetylaminobenzaldehyde thiosemicarbazone in 500 ml. of hot absolute ethanol. A yellow-green compound precipitated immediately as the pH dropped from 5.2 to 2.6. The mixture was stirred until it had cooled to room temperature and was filtered by gravity. The precipitate was washed free of sulfate ion and was air dried. The yield was 3.45 Gm. (80.8% based on a 2:1 chelate ratio).

*Anal.*—Calcd. for $C_{20}H_{22}CuN_8O_2S_2 \cdot 2H_2O$: Cu, 11.15; $H_2O$, 6.32. Found: Cu, 11.83, 11.74 (by ashing); $H_2O$, 6.42 (by wt. loss on drying).

Sulfate ion was found to be absent by barium ion test. The saturated aqueous solution gave a nega-

tive test for cupric ion with ferrocyanide ion. The test for cupric ion was still negative after the chelate was suspended for one hour in dilute hydrochloric acid (pH 3.0).

*Cobalt Chelate of Methionine.*—An aqueous solution of 1.19 Gm. (0.005 mole) of cobaltous chloride hexahydrate was added slowly, with stirring, to an aqueous alkaline solution of 1.5 Gm. (0.01 mole) of DL-methionine. The pH dropped from 7.9 to 4.7. The resulting solution was made alkaline, and a pink-brown precipitate was isolated at a pH of 10.4, and was washed free of chloride ion and air dried. The yield was 1.7 Gm. (96% based on a 2:1 chelate structure). Water of hydration was absent.

*Anal.*—Calcd. for $C_{10}H_{20}CoN_2O_4S_2$: Co, 16.60. Found: Co, 17.71.

*Copper Chelate of Methionine Sulfoxide.*—To an aqueous solution of 0.825 Gm. (0.005 mole) of methionine sulfoxide [prepared by the method of Toennies and Kolb (10)] was added 0.488 Gm. (0.005 mole) of cupric hydroxide. The mixture was stirred for three hours, the excess cupric hydroxide was removed by filtration, and the filtrate was concentrated on a steam bath to near dryness. Absolute ethanol was added, and the resulting purple compound was isolated and washed with absolute ethanol. The yield, after air drying, was 0.97 Gm. (99% based on a 2:1 chelate structure). Water of hydration was absent.

*Anal.*—Calcd. for $C_{10}H_{20}CuN_2O_6S_2$: Cu, 16.22. Found: Cu, 14.78.

*Cobalt Chelate of Cystine.*—Sufficient dilute sodium hydroxide solution was added to just dissolve 2.4 Gm. (0.01 mole) of L-cystine. To this solution was added slowly with stirring 2.38 Gm. (0.01 mole) of cobaltous chloride hexahydrate in a minimum amount of water. A pink precipitate formed immediately, and the mixture jelled when addition was complete. The pH dropped from 9.8 to 6.7. The mixture was stirred for an hour, filtered, washed free of chloride ion, and air dried. The yield was 3.1 Gm. (98% based on a 1:1 chelate structure).

*Anal.*—Calcd. for $C_6H_{10}CoN_2O_4S_2 \cdot H_2O$: Co, 18.70; $H_2O$, 5.72. Found: Co, 17.20; $H_2O$, 4.03.

*Ferrous Chelate of Cystine.*—A solution of 2.4 Gm. (0.01 mole) of L-cystine was made in dilute sodium hydroxide solution to give a pH of 9.3. An aqueous solution of 2.78 Gm. (0.01 mole) of ferrous sulfate heptahydrate was added slowly, with stirring. A yellow precipitate formed as the pH dropped from 9.3 to 6.2 and changed to an orange-brown as the pH dropped to 5.6. The precipitate was filtered, washed free of sulfate ion with water, and air dried. The yield was 2.73 Gm. (93% based on a 1:1 chelate structure). Sulfate ion was found to be absent while 1.6% moisture was found on drying.

*Anal.*—Calcd. for $C_6H_{10}FeN_2O_4S_2$: Fe, 19.01. Found, Fe, 16.87.

*Copper Complex of Biotin.*—Dilute sodium hydroxide solution was added, dropwise, to an aqueous suspension of 0.88 Gm. (0.0036 mole) of *d*-biotin until solution resulted. An aqueous solution of 0.9 Gm. (0.0036 mole) of copper sulfate pentahydrate was added slowly, with stirring. The pH dropped from 8.2 to 5.0 with the formation of a green precipitate. The yield after filtering, washing, and air drying was 0.425 Gm. (39% based on a 3:2 chelate structure). Sulfate ion was found to be absent.

*Anal.*—Calcd. for $C_{20}H_{44}Cu_2N_6O_8S_2 \cdot 4H_2O$: C, 38.80; H, 5.64; Cu, 13.69; $H_2O$, 7.76. Found: C, 38.09; H, 5.81; Cu, 12.35 (by titration), 12.01 (by ashing); $H_2O$, 8.92 (by drying), 8.93 (by Karl Fischer determination).

*Copper Derivative of Phthaloylglycine.*—An aqueous solution of 1.25 Gm. (0.005 mole) of copper sulfate pentahydrate was added slowly, with stirring, to 2.05 Gm. (0.01 mole) of phthaloylglycine [prepared by the method of Billman (11)] dissolved in dilute sodium hydroxide solution to give a pH of 7.5. A blue precipitate formed with an accompanying pH drop to 4.8. The product was filtered, washed with water, and air dried. The yield was 1.78 Gm. (68% based on a 2:1 ratio). Sulfate ion was found to be absent.

*Anal.*—Calcd. for $C_{20}H_{12}CuN_2O_6 \cdot 3H_2O$: C, 45.67; H, 3.43; Cu, 12.09; $H_2O$, 10.28. Found: C, 44.63; H, 2.85; Cu, 11.44 (by titration), 11.18 (by ashing); $H_2O$, 10.11 (by drying), 10.60 (by Karl Fischer determination).

*Copper Chelate of Phthaloylglycylglycine.*—To an aqueous suspension of 0.87 Gm. (0.0033 mole) of phthaloylglycylglycine [prepared by the procedure of Emerson (12)] was added dilute sodium hydroxide solution until solution resulted. To this solution was added slowly, with stirring, 0.42 Gm. (0.0017 mole) of copper sulfate pentahydrate in water. The pH dropped from 6.0 to 5.1 and a pale blue precipitate appeared. The yield, after filtration, washing free of sulfate ion, and air drying, was 0.65 Gm. (60% based on a 2:1 chelate structure).

*Anal.*—Calcd. for $C_{24}H_{18}CuN_4O_{10} \cdot 4H_2O$: Cu, 9.66; $H_2O$, 10.95. Found: Cu, 8.77; $H_2O$, 9.98 (by drying).

During drying a color change to green occurred. Sulfate ion was found to be absent.

*Diphthaloylglycylcystine.*—Thirty-two grams of sodium bicarbonate and 9.6 Gm. (0.04 mole) of L-cystine were mixed with 300 ml. of water. To this suspension was added slowly, with stirring, 17.9 Gm. (0.08 mole) of phthaloylglycyl chloride [made by the procedure of Emerson (12)] dissolved in 400 ml. of benzene. The mixture was stirred for one hour after the addition and the aqueous phase was separated, filtered, and made acid. The precipitate was filtered and dried, and a yield of 6.35 Gm. (26%) of product melting at 152–155° was obtained. Water was found to be present in the compound.

*Anal.*—Calcd. for $C_{26}H_{22}N_4O_{10}S_2 \cdot 2H_2O$: C, 48.00; H, 4.03. Found: C, 46.53; H, 3.84.

*Copper Chelate of Diphthaloylglycylcystine.*—Dilute sodium hydroxide solution was added, dropwise, to an aqueous suspension of 2.04 Gm. (0.0032 mole) of diphthaloylglycylcystine until solution had taken place. To this was added slowly, with stirring, 0.84 Gm. (0.0033 mole) of aqueous copper sulfate pentahydrate. The pH dropped from 6.6 to 4.4 with the formation of a green precipitate, which was collected, washed free of sulfate ion, and air dried. The yield was 1.25 Gm. (57% based on a 1:1 chelate structure).

*Anal.*—Calcd. for $C_{26}H_{20}CuN_4O_{10}S_2 \cdot 3H_2O$: Cu, 8.70; $H_2O$, 7.40. Found: Cu, 8.13 (by ashing); $H_2O$, 7.41 (by drying).

*Copper Chelate of Glycyl-DL-alanine.*—A solution of 330 mg. (0.0023 mole) of glycyl-DL-alanine (Mann Fine Chemicals) in 50 ml. of water was stirred for

TABLE I.—ANTIBACTERIAL SCREENING OF THE METAL CHELATES

| Compound | Org →[a] | Zone of Inhibition, mm. A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $(PAS)_2Cu$ complex | | 11 | 8 | 11 | 3 | 3 | 4 | 6 | 16 | 4 | 3 |
| $(PAS)_2$ Cu chelate | | 8 | 6 | 7 | 3 | 3 | 4 | 3 | 9 | 2 | 3 |
| $(PAS)_2Fe$ | | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| INH Cu | | 4 | 4 | 7 | 6 | 2 | 1 | 4 | 3 | 0 | 0 |
| INH Fe | | 3 | 0 | 8 | 2 | 2 | 3 | 4 | 7 | 2 | 0 |
| INH Zn | | 3 | 4 | 8 | 4 | 2 | 2 | 7 | 7 | 3 | 0 |
| $(INH)_2Co$ | | 14 | 11 | 19 | 9 | 8 | 8 | 16 | 13 | 13 | 8 |
| $(INH)_2Cu$ | | 2 | 0 | 3 | 0 | 4 | 2 | 3 | 2 | 0 | 0 |
| $(Tibione)_2Cu$ | | 0 | 0 | 7 | 0 | 2 | 0 | 0 | 2 | 0 | 0 |
| Biotin Cu | | 8 | 7 | 12 | 9 | 7 | 8 | 6 | 10 | 7 | 9 |
| Methionine Co | | 14 | 10 | 17 | 10 | 8 | 14 | 17 | 12 | 11 | 8 |
| Cystine Fe | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cystine Co | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Methionine sulfoxide Cu | | 5 | 2 | 4 | 0 | 3 | 5 | 3 | 0 | 0 | 0 |
| Phthaloylglycine Cu | | 4 | 9 | 5 | 3 | 3 | 3 | 6 | 4 | 4 | 0 |
| Glycylalanine Cu | | 8 | 14 | 12 | 7 | 6 | 11 | 12 | 4 | 8 | 5 |
| Diphthaloylglycylcystine Cu | | 2 | 7 | 2 | 0 | 2 | 1 | 2 | 2 | 0 | 0 |
| Phthaloylglycylglycine Cu | | 3 | 2 | 2 | 0 | 3 | 1 | 2 | 2 | 2 | 5 |
| PASA | | 7 | 5 | 12 | 4 | 2 | 7 | 2 | 0 | 0 | 0 |
| INH | | 2 | 2 | 2 | 16 | 3 | 7 | 2 | 0 | 0 | 0 |
| Tibione | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CuO | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $Fe_2O_3$ | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ZnO | | 1 | 1 | 6 | 0 | 0 | 1 | 4 | 4 | 0 | 0 |

[a] Test organisms: A—*E. coli*, B—*S. marcescens*, C—*S. albus*, D—*K. pneumoniae*, E—*P. vulgaris*, F—*B. subtilis*, G—*A. faecalis*, H—*S. aureus*, I—*A. aerogenes*, J—*Ps. aeruginosa*.

three hours with an excess of cupric hydroxide. The excess cupric hydroxide was removed by filtration, and the filtrate was concentrated to near dryness on a steam bath. Absolute ethanol was added, and the resulting deep blue crystals were filtered and washed with absolute ethanol. The yield was 400 mg. (95% based on a 2:1 chelate structure).

*Anal.*—Calcd. for $C_{10}H_{15}CuN_3O_8 \cdot H_2O$: Cu, 17.07; $H_2O$, 4.84. Found: Cu, 17.85 (by ashing); $H_2O$, 7.18 (by drying).

**Antimicrobial Screening.**—The agar plate method of the U. S. Dept. of Agriculture (13) was employed with the following organisms: *Escherichia coli*, *Serratia marcescens*, *Staphylococcus aureus*, *Staphylococcus albus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Alcaligenes faecalis*, *Aerobacter aerogenes*, *Psuedomonas aeruginosa*, and *Bacillus subtilis*. The results of the screening are shown in Table I. Duplicate determinations were made, and the zones of inhibition were measured from the edge of the compound, which was insoluble in water in all cases, to the edge of growth.

**Quantitative Determinations.**—The six most promising compounds from the screening were tested quantitatively against *S. aureus* and *E. coli* by a serial tube dilution method. Each chelate (100 mg., except in the case of biotin-copper chelate, where 22 mg. was used) was added to 100 ml. of sterile distilled water and placed in an incubator at 37°. The mixtures were shaken occasionally during forty-eight hours, by which time they had become homogeneous. A molecular equivalent of the parent compounds and of the corresponding metal salts, copper sulfate, and cobaltous chloride, was also dissolved in 100 ml. of sterile distilled water. All solutions were filtered through a sterile Berkefeld filter, and 5.0 ml. of each solution was serially diluted. To all solutions was added 0.1 ml. of a 1:100 dilution of a twenty-four-hour broth culture of test organism. After inoculation, all solutions were incubated at 37°, and at intervals of one, two, four, and eight hours, a 4-mm. loopful was transferred to a culture tube containing 5 ml. of nutrient broth. These subculture tubes were then incubated for ninety-six hours at 37° and were examined visually for the presence of growth. The highest effective dilution of each chelate and control compound is shown in Table II.

## RESULTS AND DISCUSSION

As a result of the agar plate screening procedure (Table I) six of the most active chelates were selected for the determination of bacteriostatic end points by serial tube dilution in nutrient broth. The compounds so determined were the $(PAS)_2$-copper complex (4), $(PAS)_2$-copper chelate (4), $(INH)_2$-cobalt chelate (5), glycyl-DL-alanine-copper chelate, methionine-cobalt chelate, and biotin-copper chelate. There is some question whether the biotin-copper compound, isolated as a 3:2 combination of biotin to copper ion, is actually a cyclic chelate or an open complex, since none of the usual criteria for chelation serve to distinguish the two in this case. The significant growth inhibitory differences between this compound and copper ion indicate that the product is not a salt.

Examination of Table II, showing the greatest dilutions at which growth inhibition of *E. coli* and *S. aureus* in nutrient broth was noted, reveals that the $(PAS)_2$-copper complex was devoid of activity, whereas the copper chelate was active. It was less active toward *E. coli* than either PASA or copper ion, but against *S. aureus* was more active than PASA, and by the end of eight hours was approaching the activity of copper ion. The $(INH)_2$-cobalt chelate was considerably more active than INH but distinctly less active than a molar equivalent of cobalt

TABLE II.—INHIBITORY CONCENTRATIONS OF SEVERAL METAL CHELATES *in Vitro*

| Compound | Hr. → | *E. coli*, M/1,000[a] 1 | 2 | 4 | 8 | *S. aureus*, M/1,000 1 | 2 | 4 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| $(PAS)_2Cu$ complex | | ... | ... | ... | ... | .. | ... | ... | ... |
| PASA | | 1.04 | 0.13 | 0.13 | 0.13 | .. | 4.17 | 1.04 | 1.04 |
| $CuSO_4 \cdot 5H_2O$ | | 0.15 | 0.07 | 0.07 | 0.07 | .. | 0.07 | 0.07 | 0.07 |
| $(PAS)_2Cu$ chelate | | 2.60 | 2.60 | 2.60 | 0.32 | .. | 1.30 | 0.65 | 0.16 |
| PASA | | 0.65 | 0.16 | 0.16 | 0.08 | .. | 5.16 | 2.58 | 1.29 |
| $CuSO_4 \cdot 5H_2O$ | | 0.18 | 0.09 | 0.09 | 0.09 | .. | 0.05 | 0.05 | 0.05 |
| $(INH)_2Co$ | | 1.35 | 0.04 | 0.01 | 0.005 | 0.62 | 0.62 | 0.31 | 0.08 |
| INH | | 3.12 | 0.20 | 0.20 | 0.025 | .. | ... | ... | ... |
| $CoCl_2 \cdot 6H_2O$ | | 0.15 | 0.02 | 0.002 | 0.001 | .. | 0.15 | 0.04 | 0.009 |
| Glycylalanine Cu | | ... | ... | 1.38 | 0.04 | .. | 0.34 | 0.17 | 0.08 |
| Glycylalanine | | ... | ... | ... | ... | .. | ... | ... | ... |
| $CuSO_4 \cdot 5H_2O$ | | 0.09 | 0.05 | 0.05 | 0.05 | .. | 0.05 | 0.05 | 0.05 |
| Methionine Co | | ... | ... | ... | 0.01 | 0.70 | 0.02 | 0.005 | 0.005 |
| Methionine | | ... | ... | ... | 2.75 | .. | 2.75 | 0.02 | 0.01 |
| $CoCl_2 \cdot 6H_2O$ | | 0.18 | 0.05 | 0.001 | 0.001 | .. | 0.09 | 0.02 | 0.005 |
| Biotin Cu | | 0.007 | 0.003 | 0.003 | 0.003 | .. | .. | ... | 0.003 |
| Biotin | | ... | ... | ... | ... | .. | ... | ... | ... |
| $CuSO_4 \cdot 5H_2O$ | | 0.09 | 0.05 | 0.05 | 0.05 | .. | 0.04 | 0.04 | 0.04 |

[a] A dash indicates that no inhibition of growth was observed at a concentration of 100 mg. % of chelate or its molar equivalent in the case of the chelating agent and metal ion.

ion against both organisms. Glycyl-DL-alanine-copper chelate reached the activity of copper ion in eight hours, although it was much less active in the shorter periods of time where observations were made. Methionine-cobalt chelate, however, presents a different picture. Against *E. coli* it showed no effect until eight hours had passed, when it had one-tenth the activity of cobalt ion; against *S. aureus* the chelate showed greater activity than cobalt ion until eight hours had passed, when the two activities became equal. The biotin-copper complex shows a fifteen-fold greater activity against both organisms than copper ion alone.

It appears in the cases of $(PAS)_2$-copper chelate, $(INH)_2$-cobalt chelate, and glycyl-DL-alanine-copper chelate that the antibacterial action is parallel to, or at least increases with, the partial dissociation of the chelate. The activity cannot be due wholly to dissociation, however, or the $(PAS)_2$-copper complex, which is less stable than the chelate (4), would show greater activity than the chelate. The action of the methionine-cobalt chelate on the other hand does not seem to parallel that of the cobalt ion. Here, no activity is found against *E. coli* until eight hours have passed, which is true likewise for methionine itself, while the action against *S. aureus* increases with time but is greater than that of cobalt ion until the eight-hour observation period. It is improbable that the quantities of these compounds employed would affect the pH of nutrient broth sufficiently to cause different rates of dissociation of the chelates, and the growth of the two organisms does not provide different conditions of acidity (both cultures in the broth had a pH of 6.9).

In the case of the biotin-copper complex we have a clear-cut example of a complex showing decidedly greater activity than the metal ion. Against one organism, the activity does not materially increase with time, whereas against the other, no activity is seen for eight hours, at which time it equals the activity shown *vs.* the other organism.

Some evidence has therefore been obtained, in the case of the biotin and methionine complexes, that a metal chelate or complex of a nonbacteriostatic agent can exert a distinctly greater growth inhibitory effect than a molecular equivalent of the metal ion. It appears, however, in the case of the antitubercular agents, PASA and INH, as well as Tibione, that the antibacterial activity of their metal chelates is dependent in part on a partial disssociation of the chelate to give metal ion. It may then be the case that formation of a metal chelate of these agents may increase the liposolubility sufficiently to aid in the passage of the cell wall (4, 5) and the chelate then dissociates within the cell, liberating toxic components. Whether free metal ion can exist within the cellular environment (without undergoing chelation) or even in the nutrient broth employed in these experiments in sufficient quantity to exert a toxic effect is a question still unanswered.

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# The Effect of Various Ointment Bases on the Percutaneous Absorption of Salicylates I*

## Effect of Type of Ointment Base

By MOISE E. STOLAR†, G. VICTOR ROSSI, and MARTIN BARR

**An approach to the evaluation of the percutaneous absorption of drugs from ointment bases is described. Salicylic acid and sodium salicylate were selected as test drugs for incorporation into ointment bases representing each of four physical types of ointment vehicles. These ointments were applied to the intact shaved skin of the rabbit and confined by a specially designed bandage. Concentration of salicylate in the blood was determined at hourly intervals following application of the ointment. Salicylic acid was most effectively absorbed from hydrophilic ointment. The extent of absorption from the other ointments studied, in decreasing order, was: hydrophilic petrolatum containing water, petrolatum, and polyethylene glycol ointment. Sodium salicylate was also absorbed to the greatest extent from hydrophilic ointment, although the degree of absorption was considerably less than that observed with salicylic acid. On the basis of salicylate blood levels, no significant absorption of sodium salicylate occurred from ointments prepared with petrolatum, hydrophilic petrolatum containing water, or polyethylene glycol ointment.**

ALTHOUGH a significant degree of percutaneous absorption is essential to the therapeutic usefulness of relatively few drugs applied topically in the form of ointments, it is nevertheless desirable to know the extent of such absorption from the various types of ointment bases. Such information is especially important in those cases in which absorption of drugs through the skin may lead to untoward reactions, sensitization, etc.

It was the primary aim of this study to develop a standardized procedure for determining the relative degree of absorption of drugs through the skin, and for evaluating the various factors which influence percutaneous absorption.

This report is concerned with the influence of the type of ointment base on the percutaneous absorption of salicylic acid, a predominantly oil-soluble drug, and sodium salicylate, a predominantly water-soluble drug. A subsequent report will consider the effect of various surface-active agents on the absorption of these drugs from four different physical types of ointment bases.

### EXPERIMENTAL

**Preparation of Ointments.**—Salicylic acid and sodium salicylate, previously reduced to a fine powder in a ball mill and sifted through a No. 80 sieve, were incorporated in ointment bases representing each of four physical types. The bases selected were: petrolatum U. S. P. XV, an oleaginous base; hydrophilic petrolatum U. S. P. XV in which water was incorporated, a water-in-oil emulsion base; hydrophilic ointment U. S. P. XV, an oil-in-water base; and polyethylene glycol ointment U. S. P. XV, a water-soluble base. Salicylic acid ointments were prepared to contain 6% of the active ingredient and sodium salicylate ointments were prepared to contain 6.95% sodium salicylate, which is equivalent to 6% salicylic acid.

**Analysis of Salicylic Acid Content of Ointments.**—An accurately weighed sample (2 Gm.) of salicylic acid ointment was transferred to a 100-ml. beaker to which was subsequently added 25 ml. of ethyl alcohol warmed to 65° and previously neutralized with sodium hydroxide to phenolphthalein. The mixture was stirred until the ointment first melted and then congealed. The supernatant liquid was decanted and the procedure repeated. In those cases where the ointment was miscible with alcohol, the sample was dissolved in 50 ml. of neutralized alcohol. The salicylic acid content of the supernatant liquids from both extractions, or solution of the ointment base in alcohol, was determined according to the U. S. P. XV assay for salicylic acid. To compensate for possible interference from the various components in the ointment base, a blank composed of the vehicle without salicylic acid was analyzed concurrently.

**Analysis of Sodium Salicylate Content of Ointments.**—A 2-Gm. sample of sodium salicylate ointment, accurately weighed, was transferred to a 100-ml. beaker. Sodium salicylate was either extracted with two 12.5-ml. portions of water warmed to 65°, or dissolved along with the base in 25 ml. of warmed water. The extraction was achieved by alternate melting and congealing of the ointment while being constantly stirred. The sodium salicylate content of the supernatant liquids from both extractions, or the aqueous solution of the ointment, was determined according to the U. S. P. XV assay for sodium salicylate. To compensate for possible interference from the ingredients of the ointment base, a blank composed of the vehicle without sodium salicylate was analyzed concurrently.

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All salicylic acid ointments assayed to contain 5.90–6.05% salicylic acid; all sodium salicylate ointments assayed to contain 6.80–7.10% sodium salicylate, equivalent to 5.86–6.12% salicylic acid.

**Test Animal.**—Male New Zealand white rabbits weighing between 3.0 and 3.6 Kg. were used throughout the study. The animals were maintained on Purina rabbit pellets and water *ad libitum* and housed individually in temperature and humidity controlled quarters. All experiments were performed in these same quarters.

**Application of Ointment.**—Hair was removed[1] from the skin of the dorsal area between the forelegs and hind-legs on both sides of the spine twenty-four hours prior to application of the ointment.

A bandage was devised to restrict and control the area of contact between the ointment and the skin of the rabbit. The edges of an 8.35 × 14.7 cm. piece of aluminum foil were doubled over and flattened 1 cm. on each side to produce a rectangular plate measuring 6.35 × 12.7 cm. with a 1-cm. reinforced margin. An accurately weighed 7.5-Gm. sample of ointment was uniformly spread over one surface of the plate, whose opposite side was centered on a 7.5 × 20 cm. strip of adhesive tape. The entire assembly was then applied to the shaved skin of the rabbit and carefully adjusted to conform to the contours of the area. To minimize contamination and insure adequate contact between the ointment and the skin, this assembly was covered with an Ace elastic bandage and fastened with strips of gauze bandage around the forelegs and hind-legs of the animal. The various components of the bandage are illustrated in Fig. 1, and the appearance of the bandage applied to the rabbit's skin is illustrated in Fig. 2. The ointment remained in contact with the skin for the nine-hour experimental period.

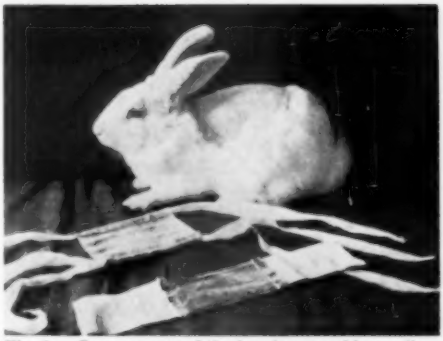

Fig. 1.—Components of the bandage used in application of test ointments to the shaved rabbit's skin.

Fig. 2.—Appearance of bandage assembly in position on the rabbit.

**Procedure.**—Due to time limitations, not more than seven animals could be utilized during any one experimental day. Therefore, on each of ten consecutive experimental days, ointments containing either salicylic acid or sodium salicylate prepared with the various type ointment bases were studied together with a salicylic acid ointment prepared from hydrophilic ointment U. S. P. XV, as a control.

One and one-half milliliters of blood were withdrawn from the marginal ear vein of the rabbit prior to, and at hourly intervals for nine hours after application of ointment. The blood samples were allowed to clot at room temperature and were then chilled in a refrigerator. After centrifugation at 2,500 r. p. m. for thirty minutes, the serum was analyzed for salicylate according to the method described by Ungar, *et al.* (1). The results obtained from a group of ten animals treated with each ointment base were analyzed by analysis of variance and the "t" test (2).

## RESULTS

The relative extent of absorption through the intact rabbit skin of salicylic acid incorporated in hydrophilic ointment, petrolatum, hydrophilic petrolatum containing water, and polyethylene glycol ointment, as determined by blood salicylate concentration (results expressed as mg. % salicylic acid), is presented in Fig. 3. The results of a similar study conducted with sodium salicylate are illustrated in Fig. 4. Each point on the graph represents an average of the determinations on groups of ten rabbits.

On the basis of the mean hourly blood levels of salicylate, as reported in Figs. 3 and 4, it is apparent that the type of ointment base exerts a definite influence on the extent of percutaneous absorption of both salicylic acid and sodium salicylate. It may also be noted, however, that the effect of the ointment base is considerably greater in regard to the absorption of salicylic acid as compared to sodium salicylate.

Salicylic acid was absorbed through the skin to a greater extent from hydrophilic ointment than from the other types of bases investigated. The extent of absorption of salicylic acid from the ointment bases studied, in decreasing order, was found to be: hydrophilic ointment, hydrophilic petrolatum, and petrolatum. Statistical analyses indicated a significant difference among the average blood salicylate concentrations obtained after application of each of these ointments. As measured by blood salicylate level, the degree of absorption of salicylic acid from polyethylene glycol ointment was negligible.

According to the criterion employed in this study, percutaneous absorption of salicylic acid and sodium salicylate occurred maximally between the fourth and sixth hour after application of the ointment to the skin of the experimental animal. It is, of course,

[1] Oster electric animal clipper, model A-2, Ang-Ra No. 2 head.

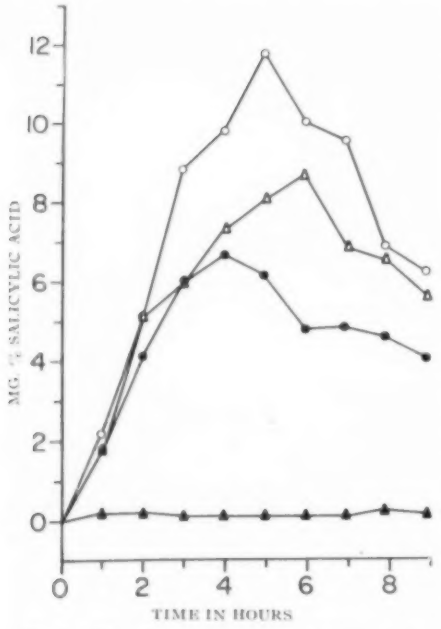


Fig. 3.—The effect of the type of ointment base on the percutaneous absorption of salicylic acid. ○, hydrophilic ointment; △, hydrophilic petrolatum with water; ●, petrolatum; ▲, polyethylene glycol ointment.

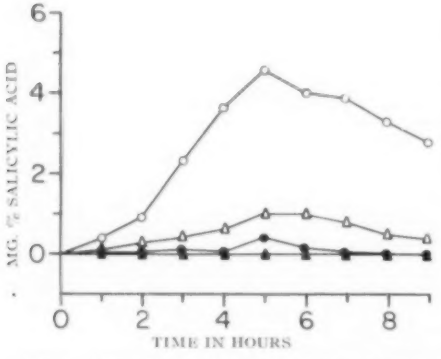


Fig. 4.—The effect of the type of ointment base on the percutaneous absorption of sodium salicylate. ○, hydrophilic ointment; ●, hydrophilic petrolatum with water; △, petrolatum; ▲, polyethylene glycol ointment.

recognized that the blood salicylate level at any given time represents a complex interaction of such factors as absorption, storage, biotransformation, and excretion.

Sodium salicylate was found to be absorbed through the skin to a measurable degree when incorporated in hydrophilic ointment, although the extent of absorption was considerably less than that observed with salicylic acid ointments prepared with this base. Statistical analyses of blood salicylate concentrations indicated that there was no significant absorption of sodium salicylate from ointments compounded with petrolatum, hydrophilic petrolatum containing water, or polyethylene glycol ointment.

## DISCUSSION

**Method.**—The procedure developed in this investigation eliminates certain variables inherent in previous methods for studying percutaneous absorption, which may, in part, be responsible for the numerous contradictory statements regarding drug absorption to be found in the literature. For example, inunction of the ointment base, which is commonly employed in absorption studies, was avoided in this investigation since it is obviously difficult to control adequately the force of application and surface area involved.

Following a study of dermal permeability in chronically heat- and humidity-stressed rats, Clay and Nelson (3) suggested that the dermal permeability of drugs may be altered by stress. To minimize the occurrence of a situation which might possibly be considered as stressful, in this study all animals were conditioned to the experimental routine twice weekly for a period of one month prior to performance of the actual experiments and were not, at any time, placed in a restraining device.

Previously reported quantitative methods for studying percutaneous absorption have generally consisted of a one-point determination of test compound in either urine or blood collected at a specified interval after application of the ointment. The technique employed in this investigation, whereby blood salicylate concentration was determined at hourly intervals for an extended period of time following application of the ointment, was thought to enable a more valid comparison of the extent of percutaneous absorption of medicament from various ointment bases.

**Solubility of Drugs.**—With the ointment bases utilized in this study it was found that salicylic acid, a predominantly oil-soluble drug, was substantially absorbed through the skin and was absorbed to a much greater extent than sodium salicylate, a predominantly water-soluble compound.

It is the opinion of many investigators in this field that oil-soluble substances are absorbed through the skin, whereas water-soluble or oil-insoluble substances are not or only poorly absorbed, since the skin is largely impermeable to water and therefore to water-soluble compounds.

The percutaneous absorption of oil-soluble substances is often quite rapid and the extent of absorption appears to increase in those cases where the compound possesses a degree of water solubility in addition to oil solubility. Despite extensive reports in regard to the influence of oil and water solubility on percutaneous absorption (4–7), it has not been established that the percutaneous absorption of compounds is a function of their distribution coefficients (i. e., oil solubility/water solubility).

Bischler, *et al.* (8), refute the necessity of water solubility in regard to the absorption of compounds through the skin. They demonstrated excellent percutaneous absorption of oleates of certain alkaloids which they state to be "insoluble in water."

On the other hand, it has been shown by the use of immunologic procedures that oil-insoluble antigens are absorbed from either ointments or wet-packs (9–12), although the degree of absorption is admittedly small.

Considerable emphasis has been placed on the importance of oil solubility, since it is believed that this property tends to enhance percutaneous absorption by the transfollicular route (13). In contrast, relatively little attention has been directed to the influence of the solubility of the drug in the ointment vehicle on the extent of percutaneous absorption. This factor will be considered in a subsequent publication.

It remains equivocal as to whether sodium salicylate is absorbed through the intact skin as such or only after conversion to salicylic acid. A study by Schwenkenbecher (14) in 1904 indicated that salicylates *per se* were not absorbed percutaneously. This conclusion was drawn from an experiment in which salicylate but not lithium ion was found in a urine sample collected twenty-four hours after the topical application of a 2% lithium salicylate solution. Schwenkenbecher, therefore hypothesized that salicylate salts were converted in the skin to salicylic acid which was subsequently absorbed and excreted in the urine. More recent renal excretion studies (15) indicate, however, that the lithium ion does not appear in the urine until two to three days after the oral ingestion of lithium chloride.

Aqueous solutions of sodium salicylate (6.95%), as well as sodium salicylate ointments having a continuous aqueous phase, were found to have a pH range of 6.2 to 6.5. The pH of the skin of the rabbit was determined to range from 6.0 to 7.4, values which are in agreement with those previously reported (16). Therefore, it is doubtful that the concentration of free salicylic acid present at such pH values could account for the amounts detected in the blood of rabbits examined during this investigation.

**Effect of Type of Ointment Base.**—The results obtained in the study of percutaneous absorption of salicylic acid from various types of ointment bases, agree, in general, with the findings of Monocarp (17, 18) who demonstrated that salicylic acid was absorbed to a greater extent from an oil-in-water emulsion base than from a water-in-oil emulsion type base or an oleaginous base. These data are futher substantiated by the results of a clinical study conducted by Polano, *et al.* (19), who demonstrated by a series of patch tests that the degree of skin irritation produced by 20% salicylic acid ointments was substantially greater with an oil-in-water emulsion vehicle than with a water-in-oil emulsion base. These investigators found no evidence of irritation following topical application of salicylic acid incorporated in polyethylene glycol ointment.

The influence of the ointment base on the extent of absorption of sodium salicylate was less distinct than in the case of salicylic acid, inasmuch as a measurable degree of absorption of sodium salicylate was observed only when hydrophilic ointment was used as the vehicle. Application to the rabbit's skin of sodium salicylate ointments prepared with petrolatum, hydrophilic petrolatum containing water, or polyethylene glycol ointment, was not followed by statistically significant blood salicylate levels. The possibility remains, however, that some degree of sodium salicylate absorption did occur from the latter three ointments, which could not be detected on the basis of blood salicylate level. Evidence for this assumption may be based on the known biologic properties of salicylates, and the indicative but not statistically significant blood salicylate concentrations obtained with petrolatum and hydrophilic petrolatum ointments.

Since the literature in regard to percutaneous absorption of sodium salicylate is meager, it was not possible to corroborate the results obtained in this study. However, Kionka (20) and Miyazaki (21) reported the absorption through the skin of sodium salicylate incorporated in lanolin, a water-in-oil emulsion type base, and petrolatum containing olive oil. These authors did not note any significant difference in the extent of absorption from the two types of ointment bases. Valette and Cesar (22) demonstrated the superiority of an oil-in-water type base to other liquid vehicles with respect to the percutaneous absorption of sodium salicylate.

## SUMMARY

1. A procedure, utilizing the intact rabbit skin, is described for the study of percutaneous absorption of drugs.

2. The relative extent of percutaneous absorption of salicylic acid and sodium salicylate from four different physical types of ointment bases was investigated.

3. The influence of the type of ointment base on the absorption of salicylic acid and sodium salicylate through the skin is considered.

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# The Effect of Various Ointment Bases on the Percutaneous Absorption of Salicylates II*

## Effect of Surface-Active Agents

By MOISE E. STOLAR†, G. VICTOR ROSSI, and MARTIN BARR

**The effect of the inclusion of several concentrations of surface-active agents in four different types of ointment bases on the percutaneous absorption of salicylic acid and sodium salicylate was determined. Hydrophilic ointment, petrolatum, hydrophilic petrolatum containing water, and polyethylene glycol ointment were modified either by varying the concentration of surface-active agent present in the U. S. P. XV formula, or by adding varying amounts of surface-active agents to those bases in which none was originally present. Sorbitan monostearate, polyoxyethylene 20 sorbitan monostearate, and polyoxyethylene 40 stearate were studied as representative of lipophilic and hydrophilic surface-active agents of the nonionic type. The results indicate a correlation between percutaneous absorption and the degree of interaction of the polyoxyethylene groups present in the hydrophilic surface-active agents with the salicylates.**

THE CURRENT CONCEPT of the role played by the ointment base in percutaneous absorption is that it facilitates contact between the medicament and the absorbing cells of the sebaceous glands and hair follicles. Bliss (1) has indicated that the properties of the drug itself, rather than the ointment vehicle, are the major determining factors in absorption from the skin. The vehicle may, however, modify the absorption properties of drugs which are capable of penetrating the skin.

Surface-active agents provide a means of improving ointment bases and enhancing the percutaneous absorption of drugs, as indicated by Dodd, *et al.* (2), by promoting diffusion of the medicament from the vehicle, or as suggested by Sweet (3), by emulsifying the sebum.

Although it is the opinion of several investigators (2–5) that surface-active agents increase the absorption of compounds incorporated in topical preparations, there are relatively little *in vivo* experimental data to substantiate this hypothesis. The present study was instituted to provide additional information in reference to the influence of surface-active agents on the absorption of drugs from ointment bases. The factors specifically considered were the effects of several surfactants, incorporated in various concentrations in four different physical types of ointment bases, on the percutaneous absorption of salicylic acid, a primarily oil-soluble drug, and sodium salicylate, a predominantly water-soluble compound.

Petrolatum U. S. P. XV; hydrophilic petrolatum U. S. P. XV, to which water was added; hydrophilic ointment U. S. P. XV; and polyethylene glycol ointment U. S. P. XV were modified either by varying the concentration of surface-active agent indicated in the U. S. P. formula, or by adding various concentrations of different surfactants to those bases in which none was originally present. Sorbitan monostearate (Span 60),[1] polyoxyethylene 20 sorbitan monostearate (Tween 60),[1] and polyoxyethylene 40 stearate (Myrj 52)[1] were selected as representative of lipophilic and hydrophilic surface-active agents of the nonionic type.

In order to elucidate the results obtained with ointments prepared from bases containing polyethylene glycols or surface-active agents of the polyoxyethylene sorbitan fatty acid ester type or the polyoxyethylene esters of fatty acid type, the effect of such substances on the solubility of salicylic acid and sodium salicylate in aqueous solutions was studied.

### EXPERIMENTAL

The experimental procedure and methods of analysis for salicylic acid and sodium salicylate in the ointment bases and in blood were previously described (6).

**Preparation of Ointment Bases.**—The various modifications of petrolatum and polyethylene glycol ointment were prepared by adding specified amounts (see Table I) of either Span 60 or Tween 60 to these bases warmed to 70°. The 70° temperature was maintained until the surface-active agents were either dissolved or dispersed in the melted base which was then allowed to cool to room temperature with constant stirring.

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[1] Atlas Powder Co., Wilmington, Del.

TABLE I.—CONCENTRATION OF SURFACE-ACTIVE AGENTS PRESENT IN OINTMENT BASES

| Surface-Active Agent | Petrolatum | Hydrophilic Petrolatum[a] | Hydrophilic Ointment | Polyethylene Glycol Ointment |
|---|---|---|---|---|
| Sorbitan monostearate | 1, 5, 10% | 1, 5, 10%[b] | ... | 1, 5, 10% |
| Polyoxyethylene 20 sorbitan monostearate | 1, 5, 10% | ... | 3, 5, 10%[c] | 1, 5, 10% |
| Polyoxyethylene 40 stearate | ... | ... | 3, 5, 10% | ... |

[a] 15 Parts purified water added to 100 parts of base.
[b] Cholesterol omitted from U. S. P. XV formula.
[c] Polyoxyethylene 40 stearate omitted from U. S. P. XV formula.

The modifications of hydrophilic ointment and hydrophilic petrolatum, incorporating various concentrations of surfactants, were prepared essentially in accordance with the directions given in the corresponding U. S. P. XV monographs. To 100 parts of hydrophilic petrolatum, and each of its variations, were added 15 parts of purified water, warmed to 72°.

Salicylic acid ointments containing 6% of the active ingredient, and sodium salicylate ointments, containing 6.95% of the sodium derivative (equivalent to 6% salicylic acid) were prepared in the manner previously described (6).

**Solubility Studies.**—The solubilities of salicylic acid and sodium salicylate in aqueous solutions containing varying amounts of polyethylene glycol 400, Tween 60, and Myrj 52, were determined. A spectrophotometric method was used for all analytical determinations (7).

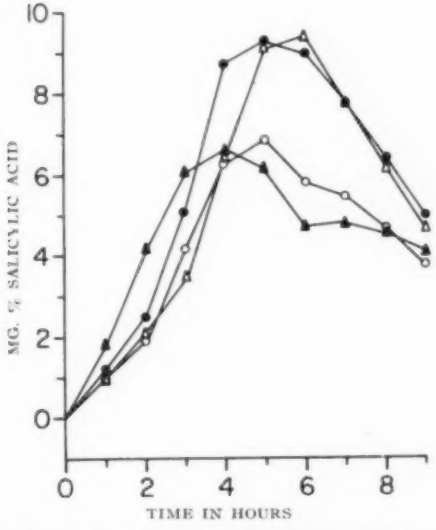


Fig. 1.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in petrolatum on the percutaneous absorption of salicylic acid. ▲, petrolatum; polyoxyethylene 20 sorbitan monostearate: ○, 1%; △, 5%; ●, 10%.

## RESULTS

The results obtained in this investigation indicate that the presence of surface-active agents in ointment bases may increase, decrease, or have no effect

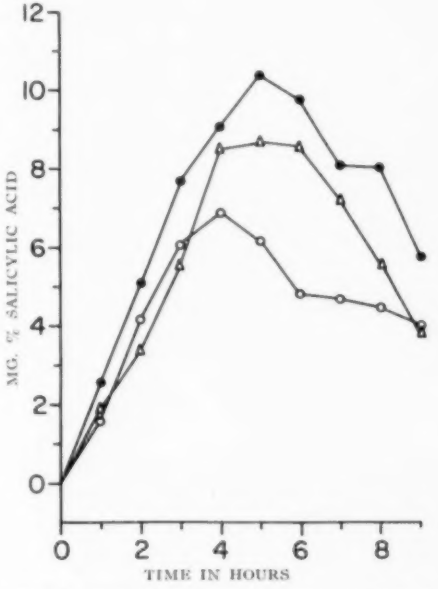


Fig. 2.—The effect of various concentrations of sorbitan monostearate in petrolatum on the percutaneous absorption of salicylic acid. Sorbitan monostearate: ○, 1%; △, 5%; ●, 10%.

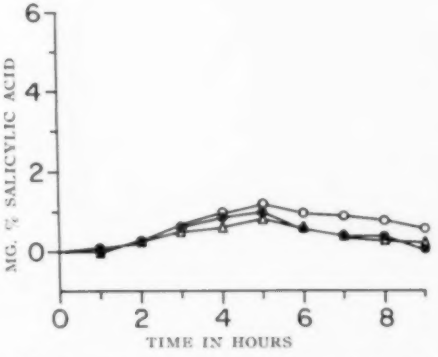


Fig. 3.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in petrolatum on the percutaneous absorption of sodium salicylate. Polyoxyethylene 20 sorbitan monostearate: ○, 1%; △, 5%; ●, 10%.

on the extent of absorption of salicylic acid and sodium salicylate through the intact rabbit skin, depending primarily on the properties of the surfactant and the ointment base.

The addition of Tween 60 or Span 60 to petrolatum had no effect on the extent of percutaneous absorption of salicylic acid when these surfactants were present in a concentration of 1%, as may be seen in Figs. 1 and 2. A marked increase in drug absorption occurred in all cases when the concentration of these surfactants was increased to 5 or 10%, however, there was no significant difference either between the effects produced by the two different surface-active agents or between the 5 and 10% concentrations.

Addition of either 1, 5, or 10% of Tween 60 to petrolatum had no effect on the percutaneous absorption of sodium salicylate (Fig. 3). The incorporation of Span 60 in concentrations of 1, 5, and 10% in petrolatum, in each case, resulted in a marked increase in the extent of sodium salicylate absorption (Fig. 4), however there was no significant difference among the effects produced by the different concentrations.

Span 60 incorporated in 5 or 10% concentrations in hydrophilic petrolatum, to which water was added, did not alter the extent of percutaneous absorption of salicylic acid or sodium salicylate as compared to the absorption of these compounds from the base without the inclusion of the surfactant (Figs. 5 and 6). A decrease in the absorption of salicylic acid was observed upon the addition of increasing concentrations of Tween 60 (Fig. 7) and Myrj 52 (Fig. 8) to hydrophilic ointment, however, the differences in blood salicylate levels obtained with the 3 and 5% concentrations of both surfactants were not statistically significant. There was no comparable reduction in the extent of absorption when these bases were employed as vehicles for sodium salicylate (Figs. 9 and 10).

On the basis of the blood salicylate level, neither salicylic acid nor sodium salicylate was absorbed to a significant extent through the skin of the rabbit when these compounds were incorporated in polyethylene glycol ointment (6). Furthermore, the inclusion of various concentrations of either Myrj 52 or Tween 60 did not lead to a significant degree of absorption of salicylic acid or sodium salicylate from polyethylene glycol ointment (7).

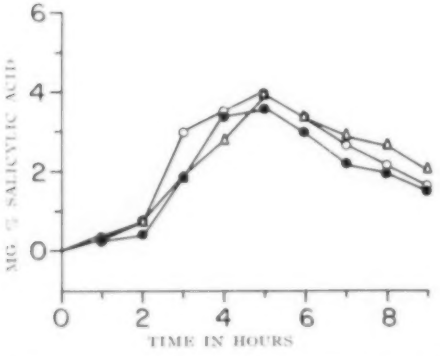


Fig. 4.—The effect of various concentrations of sorbitan monostearate in petrolatum on the percutaneous absorption of sodium salicylate. Sorbitan monostearate: ○, 1%; △, 5%; ●, 10%.

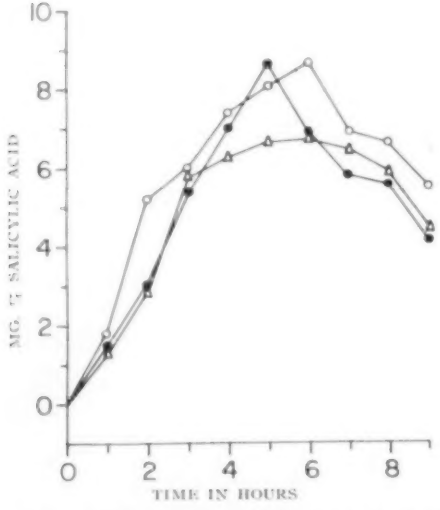


Fig. 5.—The effect of various concentrations of sorbitan monostearate in hydrophilic petrolatum containing water on the percutaneous absorption of salicylic acid. Hydrophilic petrolatum containing water, ○; sorbitan monostearate: △, 5%; ●, 10%.

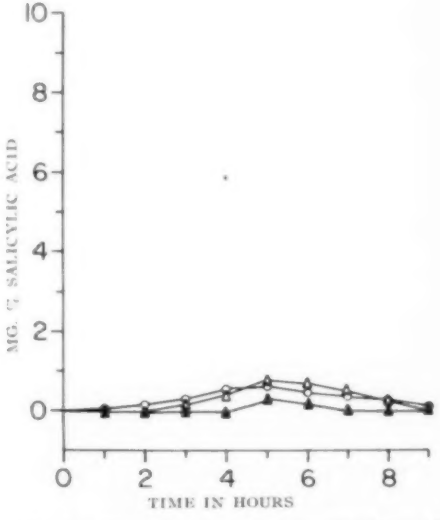


Fig. 6.—The effect of various concentrations of sorbitan monostearate in hydrophilic petrolatum containing water on the percutaneous absorption of sodium salicylate. Hydrophilic petrolatum containing water, ▲; sorbitan monostearate: ○, 5%; △, 10%.

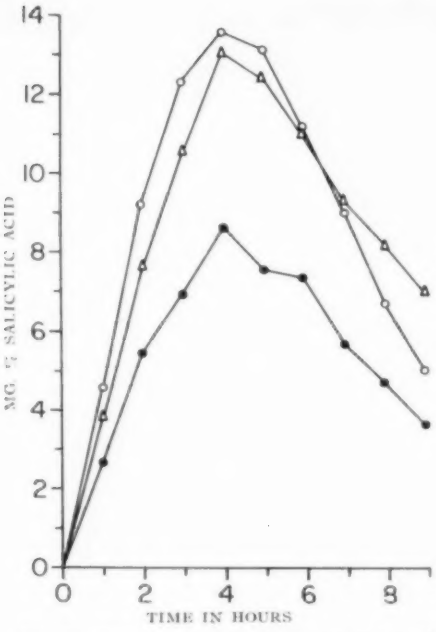


Fig. 7.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in hydrophilic ointment on the percutaneous absorption of salicylic acid. Polyoxyethylene 20 sorbitan monostearate: ○, 3%; △, 5%; ●, 10%.

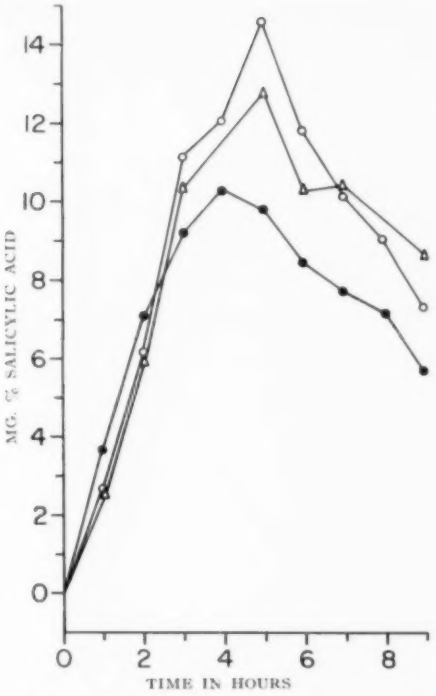


Fig. 8.—The effect of various concentrations of polyoxyethylene 40 stearate in hydrophilic ointment on the percutaneous absorption of salicylic acid. Polyoxyethylene 40 stearate: ○, 3%; △, 5%; ●, 10%.

## DISCUSSION

One of the most interesting observations made during this study was that when substances containing polyoxyethylene groups were present in ointment bases, a marked reduction in the extent of percutaneous absorption of salicylic acid resulted. This was demonstrated in studies performed with hydrophilic ointment containing Tween 60 and Myrj 52, and in the experiments utilizing polyethylene glycols.

Numerous papers have noted the interaction of compounds possessing phenolic hydroxyl groups with substances containing polyoxyethylene groups (8–14). Salicylic acid, in particular, has been shown to interact with polyoxyethylene groups (13). Therefore, it may be assumed that the reduction in the absorption of salicylic acid from ointment bases containing substances having polyoxyethylene groups is due to an interaction between these groups and the phenolic hydroxyl group of salicylic acid. The degree of interaction was assessed by determining the aqueous solubilities of salicylic acid and sodium salicylate in the presence of various concentrations of polyethylene glycol 400, Tween 60, and Myrj 52.

It was found that an increase in the concentration of the surface-active agents containing polyoxyethylene groups increased the aqueous solubility of salicylic acid (Table II). In addition, the solubility of salicylic acid increased progressively with an increase in the concentration of polyethylene glycol

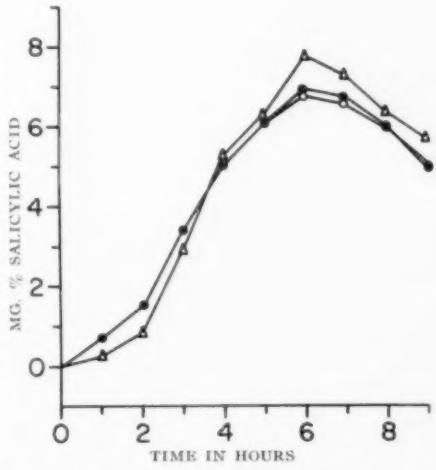


Fig. 9.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in hydrophilic ointment on the percutaneous absorption of sodium salicylate. Polyoxyethylene 20 sorbitan monostearate: ○, 3%; △, 5%; ●, 10%.

TABLE II.—SOLUBILITY (25°) OF SALICYLIC ACID IN WATER[a] CONTAINING VARIOUS CONCENTRATIONS OF POLYETHYLENE GLYCOL 400, TWEEN 60, OR MYRJ 52

| Polyethylene Glycol 400, % w/v | Salicylic Acid in Solution, Gm./100 ml. | Tween 60, % w/v | Salicylic Acid in Solution, Gm./100 ml. | Myrj 52, % w/v | Salicylic Acid in Solution, Gm./100 ml. |
|---|---|---|---|---|---|
| 0 | 0.23 | 0 | 0.23 | 0 | 0.23 |
| 1 | 0.25 | 1 | 0.37 | 1 | 0.37 |
| 3 | 0.27 | 3 | 0.61 | 3 | 0.60 |
| 6 | 0.31 | 5 | 0.86 | 5 | 0.87 |
| 10 | 0.41 | 7 | 1.06 | 7 | 0.94 |
| 15 | 0.49 | 10 | 1.47 | 10 | 1.43 |
| 20 | 0.60 | ... | ... | ... | ... |
| 30 | 1.16 | ... | ... | ... | ... |
| 40 | 2.10 | ... | ... | ... | ... |
| 50 | 6.67 | ... | ... | ... | ... |

[a] Sulfuric acid added to all solutions to produce a final concentration of 0.003 *N* $H_2SO_4$.

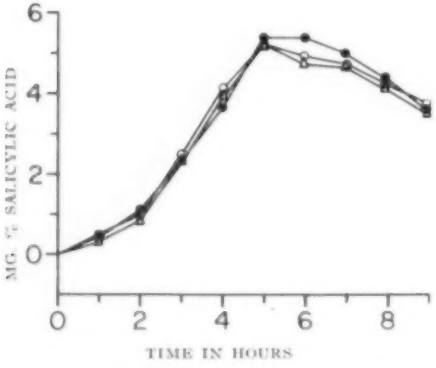


Fig. 10.—The effect of various concentrations of polyoxyethylene 40 stearate in hydrophilic ointment on the percutaneous absorption of sodium salicylate. Polyoxyethylene 40 stearate: ○, 3%; △, 5%; ●, 10%.

400 in the aqueous medium, whereas the solubility of sodium salicylate remained almost unchanged in the presence of substances containing polyoxyethylene groups (7).

Various explanations have been advanced to account for the improved solubility of substances in the presence of nonionic surfactants containing polyoxyethylene groups (15, 16). Regardless of the mechanism involved in the interaction of salicylic acid and the surface-active agents, from the results of this study, it appears that a relationship may exist between the percutaneous absorption of salicylic acid and its tendency to associate with polyoxyethylene groups.

Although the solubility of salicylates in sebum was not determined in this study, it may be theorized that one of the factors influencing the percutaneous absorption of a drug is its solubility in the vehicle as compared to its solubility in sebum. A distribution coefficient principle may be operative, i. e., the greater the solubility of the drug in the vehicle as compared to the sebum, the less the absorption through the intact skin.

The aqueous solubility of salicylic acid was found to be improved in the presence of compounds containing polyoxyethylene groups. Since emulsion bases contain water either as a continuous or as a dispersed phase, and since they also contain surfactants, the solubility of the drugs dispersed in such bases varies depending upon the specific surface-active agent present and its concentration. Such differential solubility, may, at least partly, account for the results obtained in this investigation as well as the results reported by other investigators.

## SUMMARY

1. The effect of the presence of various surface-active agents in petrolatum, hydrophilic petrolatum, hydrophilic ointment, and polyethylene glycol ointment, on the absorption of salicylic acid and sodium salicylate through the intact rabbit skin was investigated.

2. A concept is presented which relates the extent of percutaneous absorption of a drug to its relative solubility in sebum and the vehicle in which it is incorporated.

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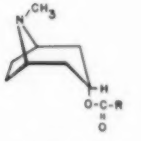
# The Synthesis of Some Glycidic Esters as Potential Antispasmodics*

By GARY OMODT† and OLE GISVOLD

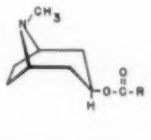
**When tropine is esterified with an appropriate acid, an active antispasmodic results. In order to study a change in the linkage between the nitrogen ring system and the acid moiety, a series of glycidic esters was prepared utilizing tropinone as the starting ketone in the Darzens condensation. Another series was prepared utilizing 1-methyl-4-piperidone.**

PREVIOUS PUBLICATIONS (1–5) indicate that tropeines are, in general, more active as antispasmodics than pseudotropeines. If a Fisher-Taylor-Hirschfelder model of a tropeine is compared to that of a pseudotropeine, it can be seen that the distance between the N-methyl and the carbonyl of the tropeine differs significantly from the corresponding distance in the pseudotropeine. Presumably, the N-methyl-carbonyl distance in a tropeine is more favorable for competition with acetylcholine than is the N-methyl-carbonyl distance in a pseudotropeine. This could account for the difference in activity.

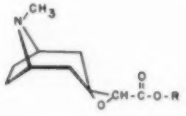
If tropinone is condensed with ethyl chloroacetate and the product transesterified with an appropriate alcohol, a compound will be obtained that would structurally resemble both a tropeine and a pseudotropeine and yet would possess an N-methyl-carbonyl distance differing from both. The purpose of this study is to determine if this would be beneficial or detrimental to antispasmodic activity.



A TROPEINE



A PSEUDOTROPEINE



A GLYCIDIC ESTER

It can be seen that the conformation of the epoxide ring would affect the N-methyl-carbonyl distance and thus the activity of the compound. Nevertheless, no attempt was made to determine the isomeric content of the compounds prepared.

A preliminary pharmacological evaluation indicated that this type of glycidic ester possessed considerable antispasmodic activity (see Fig. 1).

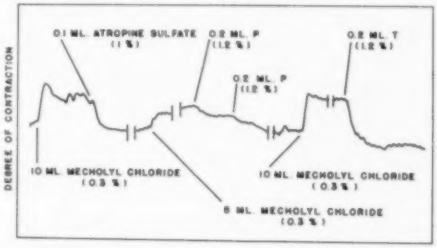

0.1 ML. ATROPINE SULFATE (1 %)
0.2 ML. P (1.2 %)
0.2 ML. T (1.2 %)
0.2 ML. P (1.2 %)
DEGREE OF CONTRACTION
10 ML. MECHOLYL CHLORIDE (0.3 %)
10 ML. MECHOLYL CHLORIDE (0.3 %)
5 ML. MECHOLYL CHLORIDE (0.3 %)

Fig. 1.—Pharmacological evaluation of benzohydryl 6-methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate hydrochloride (*P*) and benzohydryl 5,8 - methylimino - 1 - oxa - spiro - (2,6) - nonane-2-carboxylate hydrochloride (*T*).

The alcohols that were used in the transesterifications were: benzohydrol, dicyclohexylmethanol, cyclohexanol, and 1-methyl-4-piperidinol. Benzohydrol was chosen in an attempt to simulate the configuration of diphenylacetyl tropeine (an active antispasmodic). Cyclohexanol was used to determine the effect of size of the alkyl portion on the activity, and 1-methyl-4-piperidinol (assuming the cyclohexyl esters to be active) was chosen to see what effect a basic notrogen, located equidistant on each side of the carbonyl, would have on activity. Quaternization of the product, obtained when transesterifying with this last alcohol, should result in a compound possessing ganglionic blockade activity. The hydrochlorides of the benzohydryl glycidic esters were extremely sensitive to hydrolysis, probably due to the stability of the benzohydryl carbonium ion. An attempt was made to reduce this rapid hydrolysis rate by substituting the dicyclohexylmethyl group in place of the benzohydryl group. This would remove the possibility of formation of such a stable carbonium ion.

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## METHODS OF PREPARATION

Tropinone was prepared according to the procedure of Keagle, *et al.* (6). In the preparation of 1-methyl-4-piperidone, the method of McElvain, *et al.* (7), was used.

The Darzens condensation for the synthesis of glycidic esters employs a basic catalyst. The most commonly used catalysts are sodium ethoxide or sodamide (8), and in some cases potassium tertiary butoxide (9). Sodium hydride recently has been reported to be a useful catalyst in the Darzens condensation (10). Because sodium hydride-mineral oil suspension is so easily handled and stored, it was used as the Darzens catalyst. A general laboratory method for running a Darzens condensation was obtained from *Organic Reactions* (11).

A search of the literature indicated that dicyclohexylmethanol had been prepared by Mousseron, *et al.* (12), and also by Sabatier, *et al.* (13), via the Grignard reaction. However, the reaction conditions were not described in detail by either worker so the method of synthesis of di-*n*-butyl carbinol in *Organic Syntheses* (14) was followed.

1-Methyl-4-piperidinol could easily be produced by catalytic reduction of 1-methyl-4-piperidone in the Parr bomb (15) but an alternate method was utilized because of the excellent yields and the simplicity of reaction. This was the sodium borohydride reduction of 1-methyl-4-piperidone as described by Counsel (16) for the preparation of 2-hydroxyquinolizidine.

The syntheses of the different alkyl glycidic esters were carried out by following the method of transesterification as modified by Counsel (17). This method makes use of sodium hydride as the catalyst and was quite applicable to all of the glycidic ester syntheses attempted. The general procedure consisted of dissolving the appropriate alcohol and the ethyl glycidic ester in 100–150 ml. of anhydrous, redistilled Skellysolve C. The solution in a 500-ml., three-necked, round-bottomed flask equipped with a stirrer, Dean-Stark separator, reflux condenser, and drying tube was stirred and refluxed. A catalytic amount of sodium hydride suspension in mineral oil then was added to the refluxing solution. At this point, the reaction mixture became turbid and yellow colored. Reflux was continued, with stirring, for twenty hours, after which the Skellysolve C was removed by distillation under reduced pressure. When 15–20 ml. of material remained in the distilling flask, 200 ml. of ether was added and the resulting solution was washed four times with 50-ml. portions of distilled water. The washed ether solution was dried over anhydrous sodium sulfate for thirty minutes in the refrigerator. The ether was then removed by distillation under reduced pressure and the residue distilled *in vacuo*.

## BIOLOGICAL RESULTS

The pharmacological evaluation of the first two glycidic esters that were prepared was attempted in order to obtain some indication as to whether the glycidic esters of the type described might possess some antispasmodic activity. Since quite positive results were obtained (see Fig. 1), other potentially active esters were synthesized. It should be admitted, however, that only one evaluation was run on each compound and this cannot be taken as good pharmacological evidence.

## EXPERIMENTAL

All melting points recorded in this paper were taken by using a capillary tube melting point apparatus. The melting points are all uncorrected. The elemental analyses were performed by the microanalytical laboratory at the Department of Chemistry, University of Minnesota.

**Ethyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate (I) and Its Picrate.**—A solution of 48.8 Gm. (0.43 mole) of 1-methyl-4-piperidone and 53 Gm. (0.43 mole) of ethyl chloroacetate in 350 ml. of anhydrous toluene was placed in a one-liter, three-necked, round-bottomed flask equipped with a stirrer, thermometer, reflux condenser, and drying tube. The flask previously had been dried by heating with a flame while being swept out with a stream of dry nitrogen. The entire reaction was carried out under a stream of dry nitrogen. The reactants were cooled to 5° and 30.9 Gm. (0.69 mole) of a 53.7% suspension of sodium hydride in mineral oil was added as rapidly as possible while maintaining the temperature at 5° with a dry ice-acetone bath. After the addition, commercial absolute ethanol (50 drops) was added, dropwise, to the stirred mixture while the temperature was maintained at 5°. After the effervescence had subsided, the mixture was allowed to warm to room temperature and was stirred overnight. Glacial acetic acid (41.5 Gm., 0.69 mole) was then added while stirring and cooling in an ice bath. Enough water was added to dissolve the sodium acetate and the aqueous layer was removed and neutralized in the cold with potassium carbonate. The toluene layer was extracted with six 100-ml. portions of cold 3 *N* hydrochloric acid. Each portion was neutralized immediately with potassium carbonate in the cold. All of the extracts (including the neutralized acetic acid portion) were mixed and salted out with potassium carbonate and extracted continuously with ether for three days. After drying the ether extract over anhydrous sodium sulfate for one day and removal of the ether by distillation, the residue was vacuum distilled. The yellow colored product, distilling at 85–100°/0.35 mm., weighed 61.4 Gm. (72.3% yield); $n_D^{25}$ 1.4650.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of ethanol and adding dropwise with stirring, a saturated solution of picric acid in ethanol. The picrate was recrystallized four times from an ethanol-water mixture (1:1) and dried overnight at 100° under vacuum; m.p. 174–175°.

*Anal.*—Calcd. for $C_{10}H_{17}NO_3 \cdot C_6H_3N_3O_7$: C, 44.86; H, 4.79. Found; C, 44.85; H, 4.71.

**Ethyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate (II) and Its Picrate.**—This compound was prepared by following the method of synthesis of compound I with the following modifications. Tropinone (37.6 Gm., 0.27 mole) was mixed with 33.2 Gm. (0.27 mole) of ethyl chloroacetate in 350 ml. of anhydrous toluene. The reactants were cooled to −10° with a dry ice-acetone bath and 19.3 Gm. (0.43 mole) of a 53.7% suspension of sodium hydride in mineral oil was

added as rapidly as possible. After the addition, commercial absolute ethanol (60 drops) was added dropwise to the stirred reaction mixture while maintaining the temperature at −10°. The temperature then was allowed to rise slowly while keeping the effervescence under control by raising and lowering the cooling bath. After the effervescence had subsided, the mixture was allowed to warm to room temperature and stirring was maintained for eighteen hours. Glacial acetic acid (25.9 Gm., 0.43 mole) then was added to the stirred suspension while cooling in an ice bath. The yellow colored product, distilling at 110–130°/0.3 mm., weighed 28.3 Gm. (46.5% yield); $n_D^{25}$ 1.4850.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The picrate was recrystallized four times from ethanol and dried overnight at 100° under vacuum; m.p. 154–155°.

*Anal.*—Calcd. for $C_{12}H_{19}NO_3 \cdot C_6H_3N_3O_7$: C, 47.58; H, 4.88. Found: C, 47.65; H, 5.01.

**Dicyclohexylmethanol.**—Into a three-liter, three-necked, round-bottomed flask fitted with a 500-ml. dropping funnel, a stirrer, and a reflux condenser with drying tube, was placed 36.5 Gm. (1.5 gram-atoms) of magnesium turnings and 500 ml. of absolute ether. The synthesis was carried out by following the method for the synthesis of di-*n*-butyl carbinol in *Organic Synthesis* (14) and using correct molecular proportions of additional reagents. Also, the reaction mixture was not steam distilled. The cooled mixture of carbinol and aqueous potassium hydroxide was extracted first with 200 ml. of ether and then with three additional 100-ml. portions of ether. The combined ether extracts were dried over anhydrous potassium carbonate for one hour. After removal of the ether by distillation under reduced pressure, the residue was distilled, *in vacuo*, through a fractionating column, the fraction distilling at 125–128°/4.5 mm. being the product [reported b.p. 166°/20 mm. (13)]. The yield was 89.3 Gm. (60.8%).

**1-Methyl-4-piperidinol.**—A solution of 1-methyl-4-piperidone (56.5 Gm., 0.5 mole) in 200 ml. of water was added slowly to a solution of 5.5 Gm. of sodium borohydride in 200 ml. of water. The mixture was stirred and kept cool with an ice bath during the addition. After allowing the mixture to stand at room temperature overnight, 73 ml. of concentrated ammonium hydroxide solution was added and the mixture allowed to stand for an additional hour. The solution then was saturated with anhydrous sodium sulfate. Two layers formed. The upper layer was removed and the lower layer was extracted two times with 200-ml. portions of hot benzene. The extracts were combined with the upper layer and the solvents were removed by distillation under reduced pressure. The residue was vacuum distilled, the product distilling at 85–88°/6 mm. [reported b. p. 83°/under 8 mm. (18)]. The yield was 40.5 Gm. (70.4%); $n_D^{25}$ 1.4770.

**Benzohydryl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its Hydrochloride and Picrate.**—This compound was prepared by slightly modifying the general procedure for transesterification. Benzohydrol (5.2 Gm., 0.028 mole) and 5.6 Gm. (0.028 mole) of I were dissolved in 100 ml. of anhydrous, redistilled Skellysolve C. After bringing the solution to reflux temperature and addition of the catalyst, the first ten 10-ml. portions of distillate were removed, each with the simultaneous addition of a 10-ml. portion of fresh Skellysolve C to the reaction flask. The reaction mixture was then refluxed for twenty hours and worked up in the usual manner. The yellow colored product, distilling at 200–205°/0.4 mm., weighed 4.4 Gm. (46.7% yield); $n_D^{25}$ 1.5525.

A hydrochloride was prepared by adding, dropwise with stirring, an ethereal solution of hydrogen chloride to a solution of 1 Gm. of material dissolved in 25 ml. of absolute ether. The precipitated hydrochloride was filtered under an atmosphere of dry nitrogen because it was so hygroscopic. It was recrystallized once from a mixture of commercial absolute ethanol and isopropyl ether. Further attempts at recrystallization for the purpose of preparing an analytical sample resulted in an oil which could not be recrystallized. The oil was forced to crystallize by drying under high vacuum at 100°; m. p. 173–175° (decompn.). This material was submitted, as such, for analysis.

*Anal.*—Calcd. for $C_{21}H_{23}NO_3 \cdot HCl$: C, 67.46; H, 6.47. Found: C, 59.11; H, 6.28.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from an ethanol-water mixture (3:1) and dried overnight at 100° under vacuum; m. p. 189–190°.

*Anal.*—Calcd. for $C_{21}H_{23}NO_3 \cdot C_6H_3N_3O_7$: C, 57.24; H, 4.63. Found: C, 57.33; H, 4.70.

**Dicyclohexylmethyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its *p*-Toluene Sulfonate and Picrate.**—Anhydrous dicyclohexylmethanol (5.9 Gm., 0.03 mole) and 5.9 Gm. (0.03 mole) of I were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.[1] The yellow colored product, distilling at 170–188°/0.4 mm., weighed 7.2 Gm. (68.8% yield); $n_D^{25}$ 1.4923.

A *p*-toluene sulfonate was prepared by dissolving 1 Gm. of material in 25 ml. of absolute ether and adding, dropwise with stirring, a saturated solution of *p*-toluene sulfonic acid monohydrate in absolute ether. The precipitated *p*-toluene sulfonate was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 175–176°.

*Anal.*—Calcd. for $C_{21}H_{33}NO_3 \cdot C_7H_8O_3S$: C, 64.46; H, 8.31. Found: C, 64.67; H, 8.33.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from commercial absolute ethanol and dried overnight at 100° under vacuum; m. p. 191–192° (decompn.).

*Anal.*—Calcd. for $C_{21}H_{33}NO_3 \cdot C_6H_3N_3O_7$: C, 56.04; H, 6.62. Found: C, 55.49; H, 6.60.

[1] Modified by packing with glass wool and calcium hydride so as to prevent any reflux of ethanol into the reaction mixture.

**Cyclohexyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its *p*-Toluene Sulfonate and Picrate.**—Anhydrous cyclohexanol (12.8 Gm., 0.128 mole) and 7.2 Gm. (0.064 mole) of I were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.[1] The yellow colored product, distilling at 145–155°/0.2 mm., weighed 6.3 Gm. (37.2% yield); $n_D^{25}$ 1.4828.

A *p*-toluene sulfonate was prepared by dissolving 1 Gm. of material in 25 ml. of absolute ether and adding, dropwise with stirring, a saturated solution of *p*-toluene sulfonic acid monohydrate in anhydrous ether. The precipitated *p*-toluene sulfonate was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 132–133°.

*Anal.*—Calcd. for $C_{14}H_{25}NO_3 \cdot C_7H_8O_3S$: C, 59.27; H, 7.34. Found: C, 59.35; H, 7.34.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The oily picrate was frozen in a dry ice-acetone bath and crushed with a stirring rod. The powdered picrate was recrystallized four times from methanol and dried overnight at 100° under vacuum; m. p. 139–140°.

*Anal.*—Calcd. for $C_{14}H_{25}NO_3 \cdot C_6H_3N_3O_7$: C, 49.79; H, 5.43. Found: C, 49.83; H, 5.51.

**1-Methyl-4-piperidyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its Methyl Bromide and Picrate.**—This compound was prepared by slightly modifying the general procedure for transesterification. Anhydrous 1-methyl-4-piperidinol (5.8 Gm., 0.05 mole) and 10 Gm. (0.05 mole) of I were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C. After bringing the solution to reflux temperature and addition of the catalyst, the first 25 ml. of distillate were removed and reflux was continued, with stirring, for eighteen hours, after which, the Skellysolve C was removed by distillation under reduced pressure. The residue was distilled *in vacuo*.[2] The yellow colored product, distilling at 145–155°/1 mm., weighed 6.7 Gm. (50% yield); $n_D^{25}$ 1.4876.

A methyl bromide was prepared by dissolving 1 Gm. of material in 20 ml. of anhydrous ether and adding 20 ml. of methyl bromide. The mixture was kept for three days at room temperature in a pressure bottle. The precipitated methyl bromide was filtered under dry nitrogen and recrystallized four times from a methanol-isopropyl ether mixture (filtering under dry nitrogen each time because of hygroscopicity). The hygroscopic methyl bromide was dried overnight at 100° under vacuum. A melting point could not be obtained under 270°. The compound, however, decomposed with charring over the range 189–270°.

*Anal.*—Calcd. for $C_{14}H_{25}N_2O_3 \cdot 2CH_3Br \cdot {}^1/_2H_2O$: C, 41.12; H, 6.69. Found: C, 40.99; H, 6.92.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding it, dropwise with stirring, to 20 ml. of a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from an acetone-ethanol mixture. The picrate first was dissolved in a minimum amount of acetone and an equal volume of commercial absolute ethanol was added to it. The resulting solution then was concentrated to one-half of its original volume and crystallization was allowed to take place. The recrystallized picrate was dried overnight at 100° under vacuum; m. p. 203–204° (decompn.).

*Anal.*—Calcd. for $C_{14}H_{24}N_2O_3 \cdot 2C_6H_3N_3O_7$: C, 42.98; H, 4.16. Found: C, 42.89; H, 4.35.

**Benzohydryl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its Hydrochloride and Picrate.**—Benzohydrol (10.3 Gm., 0.056 mole) and 6.3 Gm. (0.028 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.[1] The yellow colored product, distilling at 220–235°/0.1 mm., weighed 8.2 Gm. (80.6% yield); $n_D^{25}$ 1.5572.

A hydrochloride was prepared by adding, dropwise with stirring, an ethereal solution of hydrogen chloride to a solution of 1 Gm. of material dissolved in 25 ml. of absolute ether. The precipitated hydrochloride was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 165–166° (decompn.).

*Anal.*—Calcd. for $C_{21}H_{29}NO_3 \cdot HCl$: C, 69.07; H, 6.55. Found: C, 68.67; H, 6.49.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from an ethanol-acetone mixture. The picrate was dissolved in a minimum amount of acetone and an equal volume of commercial absolute ethanol was added to it. The solution was concentrated to one-half of its original volume and crystallization was allowed to take place. The recrystallized picrate was dried overnight at 100° under vacuum; m. p. 184–185° (decompn.).

*Anal.*—Calcd. for $C_{21}H_{29}NO_3 \cdot C_6H_3N_3O_7$: C, 58.78; H, 4.76. Found: C, 58.90; H, 4.96.

**Dicyclohexyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its Hydrochloride and Picrate.**—Anhydrous dicyclohexylmethanol (3.9 Gm., 0.02 mole) and 4.5 Gm. (0.02 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.[1] The yellow colored product, distilling at 185–200°/1 mm., weighed 5.2 Gm. (69.5% yield); $n_D^{25}$ 1.5001.

A hydrochloride was prepared by adding, dropwise with stirring, an ethereal solution of hydrogen chloride to a solution of 1 Gm. of material dissolved in 25 ml. of absolute ether. The precipitated hydrochloride was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 235–236° (decompn. at 190°).

*Anal.*—Calcd. for $C_{28}H_{42}NO_3 \cdot HCl \cdot H_2O$: C, 64.24; H, 9.31. Found: C, 64.57; H, 9.11.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated

[2] The residue was distilled directly without washing since a previous synthesis of the same compound indicated that it was too water-soluble to permit washing with water.

solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from absolute ethanol; m. p. 189–190°.

*Anal.*—Calcd. for $C_{25}H_{42}NO_2 \cdot C_6H_3N_3O_7$: C, 57.60; H, 6.67. Found: C, 57.90; H, 6.82.

**Cyclohexyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its *p*-Toluene Sulfonate and Picrate.**—Anhydrous cyclohexanol (5.6 Gm., 0.056 mole) and 6.3 Gm. (0.028 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.[1] The yellow colored product, distilling at 165–175°/0.5 mm., weighed 5.4 Gm. (69.3% yield); $n_D^{25}$ 1.4961.

A *p*-toluene sulfonate was prepared by dissolving 1 Gm. of material in 25 ml. of absolute ether and adding, dropwise with stirring, a saturated solution of *p*-toluene sulfonic acid monohydrate in anhydrous ether. The precipitated *p*-toluene sulfonate was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 134–135°.

*Anal.*—Calcd. for $C_{18}H_{29}NO_3 \cdot C_7H_8O_3S$: C, 61.17; H, 7.37. Found: C, 61.10; H, 7.49.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from commercial absolute ethanol and dried overnight at 100° under vacuum; m. p. 205–206° (decompn.).

*Anal.*—Calcd. for $C_{18}H_{29}NO_3 \cdot C_6H_3N_3O_7$: C, 51.96; H, 5.55. Found: C, 51.96; H, 5.55.

**1-Methyl-4-piperidyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its Methyl Bromide and Picrate.**—This compound was prepared by slightly modifying the general procedure for transesterification. Anhydrous 1-methyl-4-piperidinol (5.4 Gm., 0.047 mole) and 10.5 Gm. (0.047 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C. After bringing the solution to reflux temperature and addition of the catalyst, a portion of distillate (350 ml.) was removed while simultaneously adding, dropwise, 350 ml. of fresh Skellysolve C. Reflux was continued, with stirring, for six hours, after which the Skellysolve C was removed by distillation under reduced pressure. The residue was distilled *in vacuo*.[2] The yellow colored product, distilling at 155–175°/0.45 mm., weighed 5.1 Gm. (36.9% yield); $n_D^{25}$ 1.4962.

A methyl bromide was prepared by dissolving 1 Gm. of material in 20 ml. of anhydrous ether and adding 20 ml. of methyl bromide. The mixture was kept three days at room temperature in a pressure bottle. The precipitated methyl bromide was filtered under dry nitrogen and recrystallized four times from a methanol-isopropyl ether mixture (filtering under dry nitrogen each time because of hygroscopicity). The very hygroscopic methyl bromide was dried overnight at 100° under vacuum. A melting point could not be obtained under 265°. The compound, however, decomposed with charring, over the range 230–265°.

*Anal.*—Calcd. for $C_{18}H_{31}N_2O_3 \cdot 2CH_3Br \cdot 2H_2O$: C, 41.16; H, 6.97. Found: C, 41.13; H, 7.19.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding it, dropwise with stirring, to 20 ml. of a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from an acetone-ethanol mixture. The picrate first was dissolved in a minimum amount of acetone and an equal volume of commercial absolute ethanol was added to it. The resulting solution then was concentrated to one-half of its original volume and crystallization was allowed to take place. The recrystallized picrate was dried overnight at 100° under vacuum; m. p. 227–228° (decompn.).

*Anal.*—Calcd. for $C_{18}H_{31}N_2O_3 \cdot 2C_6H_3N_3O_7$: C, 44.68; H, 4.29. Found: C, 44.72; H, 4.32.

## PHARMACOLOGICAL EVALUATION

The apparatus for the pharmacological evaluation consisted of a glass cylinder 12 cm. high with an inside diameter of 5 cm. A large rubber stopper equipped with a glass drain tube was fitted into one end of the cylinder. Rubber tubing was attached to the glass drain tube and the whole was immersed in a constant temperature bath in such a manner that the cylinder could be drained of its contents by applying suction to the rubber tubing while the cylinder remained immersed in the bath. A metal rod with a muscle hook was placed inside the cylinder and a muscle lever was situated so that its hook was located directly above the hook inside the cylinder. The muscle lever was so arranged that it would trace on a revolving kymograph drum covered with smoked kymograph paper. A glass tube, connected to a supply of oxygen, was also placed inside the cylinder so that any solution inside the cylinder could be readily oxygenated. Locke's solution (150 ml.) was placed in the cylinder and the temperature bath was warmed to 38° with a thermostatically controlled water heater. The temperature was maintained at 38 ± 0.1°. When the temperature of the Locke's solution had reached 38°, a flow of oxygen was started and a 4-cm. section of freshly excised rabbit ileum[3] was attached to the muscle hooks so that the muscle section was completely submerged in the warm Locke's solution. The kymograph drum was slowly rotated and 10 ml. of a 0.3% solution of Mecholyl chloride[4] was added to the Locke's solution. After the muscle had responded to the Mecholyl chloride, 0.1 ml. of a 1% solution of atropine sulfate was added. The same procedure was employed with the following, utilizing a fresh section of ileum and 150 ml. of fresh Locke's solution each time: 5 ml. of Mecholyl chloride (0.3%) followed by 0.2 ml. of compound *P* (1.2%), followed by an additional 0.2 ml. of compound *P* (1.2%); 10 ml. of Mecholyl chloride (0.3%) followed by 0.2 ml. of compound *T* (1.2%). All solutions were prepared by using recently boiled, distilled water as the solvent. The results of the pharmacological evaluation are shown in Fig. 1.

[3] The freshly excised rabbit ileum was stored in a cold solution of Locke's solution and rabbit blood.
[4] Trade name for methacholine chloride.

## SUMMARY

Eight new glycidic esters that may possess antispasmodic activity were prepared.

Two new glycidic esters were pharmacologically screened for their antispasmodic activity with apparently positive results.

Sodium borohydride was found to be effective in the reduction of 1-methyl-4-piperidone.

Sodium hydride was found to be a convenient and efficient base in the Darzens condensation.

Sodium hydride was found to be a good catalyst in the transesterification of the glycidic esters.


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# A Quantitative Method for the Alkaloid of *Acacia berlandieri**

By BENNIE J. CAMP and JOAN A. MOORE

**Using the reagent 1-fluoro-2,4-dinitrobenzene, a rapid and reliable method is described for the quantitative estimation of N-methyl beta-phenylethylamine in *Acacia berlandieri*. The method appears to be specific for the amine since paper chromatograms of plant extracts failed to indicate the presence of interfering amines and alkaloids.**

ACACIA BERLANDIERI (guajillo) is a leguminous plant which contains a compound poisonous to certain animals. Camp and Lyman (1) isolated and identified the poisonous principle as N-methyl beta-phenylethylamine.

It became necessary to develop a quantitative method for the determination of this sympathomimetic compound before the following studies could be made: (*a*) the effects of environmental factors such as temperature and rainfall on the seasonal variation of the amine content of the plant, (*b*) the metabolism of the compound by microorganisms, (*c*) the deposition of the amine in certain mammaliam tissues, and (*d*) the complexing of the amine with organic and inorganic compounds.

McIntire, *et al.* (2), demonstrated the use of 1-fluoro-2,4-dinitrobenzene as a quantitative reagent for certain primary and secondary amines. The purpose of this paper is to report a quantitative procedure for assaying the N-methyl beta-phenylethylamine content of *Acacia berlandieri*.

## EXPERIMENTAL

**Synthesis.**—Since this amine is not available commercially, it was necessary to synthesize the compound. To 5 L. of dry *n*-butyl alcohol containing 15 moles (465 Gm.) of anhydrous monomethylamine are added 3 moles (555 Gm.) of (2-bromoethyl) benzene. The vessel is stoppered and allowed to stand for twenty-four hours at room temperature. It is then heated on a steam bath to increase the rate of reaction. The monomethylamine hydrobromide which forms as a side product is removed by filtration, and the supernatant liquid is concentrated to approximately 25% of its original volume by *in vacuo* distillation. Upon cooling, the hydrobromide salt of the amine crystallizes from the *n*-butyl alcohol. The amine is converted to the hydrochloride salt by treating the *n*-butyl alcohol containing the amine hydrobromide salt with 40% sodium hydroxide until the alcohol phase becomes distinctly alka-

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line. The free base formed by the addition of sodium hydroxide is readily soluble in the *n*-butyl alcohol phase, and the alcohol and aqueous phases are separated by means of a separatory funnel. The *n*-butyl alcohol is dried over anhydrous sodium sulfate, and anhydrous hydrogen chloride gas is passed into the alcohol solution until it becomes distinctly acid. If the *n*-butyl alcohol is not dry before the addition of the hydrogen chloride gas, then the hydrochloride salt will contain water of crystallization which melts at a lower temperature, 141–142°. The compound is purified by dissolving in chloroform and then filtered to remove small quantities of monomethylamine hydrochloride present. The hydrochloride salt crystallizes upon the addition of anhydrous ether to the chloroform solution. The compound is stored as the acid salt because the free base decomposes upon standing. The following melting points were obtained for derivatives prepared from the synthetic compound: hydrochloride, m.p. 157–158°; reported m. p. 157–158° (3), and 161–162° (4); picrate, m. p. 139–141°; reported m. p. 141–142° (4), and 141° (5).

The free base is formed by dissolving the acid salt in concentrated sodium hydroxide; the base forms an oily layer and is separated in a separatory funnel. It is washed several times with distilled water, dried over anhydrous sodium sulfate, and distilled under reduced pressure. It boils at 107–108° at 31 mm. pressure; reported b. p. 67.4° at 3.25 mm. (2) and 112.5–115° at 36–40 mm. pressure (5).

**Standard Curve.**—The free amine is used to establish the standard curve. The procedure for preparing the 2,4-dinitrobenzene derivative is similar to the method described by McIntire, *et al.* (2). A series of standards, ranging in amine content from 10 to 100 $\gamma$, are prepared by transferring with a pipet the appropriate amount of the alcoholic solution of the amine to a series of 25-ml. volumetric flasks, each containing 0.10 ml. of 1% alcoholic FDNB (Eastman), 0.2 ml. of 0.2 *M* $NaHCO_3$, 0.2 ml. absolute alcohol, and sufficient water to make a total volume of 1.5 ml. The flask is heated in a 60° water bath for twenty minutes. The excess reagent is then hydrolyzed by adding 0.5 ml. of 0.2 *N* NaOH and incubating an additional sixty minutes in the water bath. The contents of the flask are diluted to volume and extracted with 10 ml. of cyclohexane in a separatory funnel. The absorbance of the cyclohexane phase is read on a Beckman DU spectrophotometer at 350 m$\mu$, using a blank to adjust the instrument to zero absorbance. The blank, alcohol without amine, was developed in the same manner as the alcoholic amine standards. Figure 1 represents a typical standard curve. The relationship between the absorbance of the 2,4-dinitrobenzene derivative and the quantity of the amine used in the reaction is linear when the quantity of amine does not exceed 120 $\gamma$. However, the relationship becomes curvilinear when the quantity of amine used in the reaction is greater than 120 $\gamma$, not shown on curve.

**Assay of Plant Material.**—The air dried leaves from the acacia plant are ground in a Wiley mill to pass a 20-mesh screen. Five grams of the sample are extracted with 250 ml. of 1% HCl for approximately two hours in a 1-L. wide-mouth polyethylene bottle ($3^1/_2 \times 6^1/_2$ inches) on a magnetic stirrer. The bottle and contents are centrifuged, and the clear, supernatant liquid is decanted into a 500-ml. volumetric flask. It is re-extracted twice with 100 ml. of 1% HCl for one hour, centrifuged, and diluted to volume. A 100-ml. aliquot is adjusted to pH 10 with sodium hydroxide and extracted three times each with 100 ml. of chloroform. The combined chloroform extracts are made acid with 0.5 ml. of alcoholic HCl (alcohol saturated with hydrogen chloride gas) and reduced to dryness by vacuum distillation. The residue is dissolved in absolute alcohol and transferred quantitatively to a 25-ml. volumetric flask. It is neutralized with sodium hydroxide, and diluted to volume. A 0.2-ml. aliquot sample is used in the reaction with FDNB. The quantity of the amine in the aliquot sample is found by reading graphically from the standard curve the amount of amine equivalent to the absorbance of the FDNB derivative.

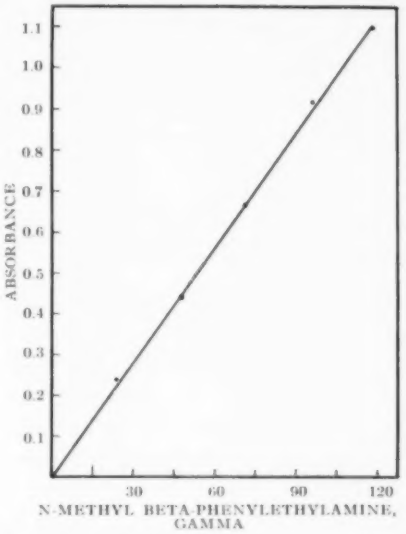


Fig. 1.—Relationship of absorbance of 2,4-dinitrobenzene derivative and quantity of amine used in reaction.

## RESULTS

Table I gives the per cent recovery when known quantities of the pure amine compound were carried through the chloroform extraction procedure and the converted to the 2,4-dinitrobenzene derivative.

TABLE I.—PER CENT RECOVERY OF AMINE COMPOUND

| Sample | Theory, gamma/0.5 ml. | Found,[a] gamma/0.5 ml. | Theory, % |
|---|---|---|---|
| 1 | 25 | 27.00 | 108.0 |
| 2 | 25 | 27.25 | 110.0 |
| 3 | 50 | 51.50 | 103.0 |
| 4 | 50 | 53.00 | 106.0 |

[a] Average of duplicate samples.

Table II presents the results of a recovery study when known quantities of the amine were added to plant extracts. An aliquot part of the sample was used which would fall within the 100 γ range. Within this range, the average per cent recovery exceeds 95% and demonstrates the reliability of this method as a quantitative procedure for the amine content of this plant.

TABLE II.—RECOVERY OF AMINE ADDED TO PLANT EXTRACT

| Sample | Found Plant, gamma/0.1 ml. | Added, gamma/0.1 ml. | Total Found, gamma | Recovered % |
|---|---|---|---|---|
| 1 | 26.5 | 25.7 | 58.0[a] | 111.0 |
| 2 | 26.5 | 41.5 | 65.0 | 95.6 |
| 3 | 26.5 | 51.9 | 74.5 | 95.2 |
| 4 | 26.5 | 62.3 | 83.0 | 93.4 |

[a] Average of duplicate samples.

To further test the reliability of this method, the month of July sample was repeatedly assayed, and the following values were obtained: 0.38, 0.44, 0.40, 0.40, 0.46, 0.42, 0.43, 0.46, 0.43, and 0.44%.

Leaf samples from the acacia plant were collected during the 1958 growing season to ascertain if any change occurred in the amine content of the plant during this period. Table III presents the results obtained from an analysis of leaf samples during 1958. A slight fluctuation occurs within the growing season, and samples are being collected for the 1959 growing season to determine if this fluctuation is correlated with environmental factors.

This method is currently being used in the author's laboratory to study the utilization of N-methyl beta-phenylethylamine by microorganisms.

TABLE III.—AMINE CONTENT OF LEAF SAMPLES FROM *Acacia berlandieri*

| Sample | Amine, %[a] |
|---|---|
| May | 0.66 |
| June | 0.46 |
| July | 0.42 |
| August | 0.46 |
| September | 0.28 |
| October | 0.46 |

[a] Per cent based on weight of air dried sample.

## SUMMARY

1. A method is described for a large scale synthesis of N-methyl beta-phenylethylamine.

2. An analytical procedure is described for assaying the sympathomimetic amine content of *Acacia berlandieri*.

3. Data are given showing the amine content of leaf samples during the 1958 growing season.

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# A Simplified Colorimetric Method for Determination of Mercury in Biologic Materials*

By DONALD L. SORBY and ELMER M. PLEIN

**A simplified colorimetric method for determination of mercury by the dithizone method has been developed. The procedure is suitable for analyzing biological and certain mineral samples for their mercury content. Amounts of mercury between 10 and 100 mcg. have been analyzed with a maximum deviation of ±5 per cent. The procedure has been simplified wherever possible to do so without sacrificing accuracy.**

FOR AN INVESTIGATION of the penetration of ammoniated mercury through the skin of laboratory animals, a simple and rapid procedure for determining mercury in biologic material was desired. Various procedures were investigated (1–6) and were found to be rather time-consuming or not of the desired selectivity. The method presented here combines the most desirable features of two of these methods (3, 4).

The digestion procedure of Polley and Miller (4) was chosen because of its simplicity and relatively mild conditions. For determination of mercury in the digest solutions the procedure of Plein and Plein (3) was utilized because this method is quite simple and requires comparatively little time to carry out.

## EXPERIMENTAL

**Reagents.**—Chloroform is distilled in glass apparatus and 0.75% ethanol is added as a preservative; sulfuric acid, concentrated and 0.5 *N*; hydro-

* Received August 17, 1959, from the University of Washington, College of Pharmacy, Seattle.

gen peroxide, 90%, solutions of approximately 50% strength are freshly prepared by dilution with distilled water; dithizone solution, diphenylthiocarbazone (Eastman white label), is dissolved (1 mg. per ml.) in chloroform and refrigerated, fresh dilutions of this solution containing 4 mg. per liter are prepared for each assay; hydrochloric acid, 0.25 *N*; hydroxylamine hydrochloride solution, 20%, purified by shaking with several portions of a chloroform solution of dithizone (10 mg. per ml.) and washing with chloroform until the green color is removed; potassium permanganate, 5%; standard mercuric chloride solution, 1,000 mcg. mercury per ml. mercuric chloride (0.1354 Gm.) is dissolved in 100 ml. of 0.5 *N* sulfuric acid, and this solution is diluted 1:100 for use in preparing a standard curve for each determination.

Laug, *et al.* (1), recommended the use of glass distilled water in order to avoid interference of copper in the assay. Water for this experiment was obtained from a Barnstead type "Q" still. Water from this source was found to be satisfactory for use in the dithizone procedure.

**Digestion of the Sample.**—The digestion procedure differs somewhat for each type of sample. Such factors as moisture content, amount of organic matter present, physical form of the sample, and presence of undigestible material require slight modifications of the following procedure to fit the particular sample.

A one-gram sample is placed in a 250-ml. flat-bottomed standard-taper flask and an Ahlin condenser is fitted to the flask. Five milliliters concentrated sulfuric acid is added through the condenser. The apparatus is heated on a steam bath and 50% hydrogen peroxide is added, dropwise, through the condenser with swirling of the sample. The dropwise addition of the hydrogen peroxide is continued until the solution becomes clear and nearly colorless. The steam bath is then removed and the flask is heated on an asbestos pad over a low flame. Drops of 50% hydrogen peroxide are added and heating is continued as the solution darkens. Heating is discontinued when the samples remain clear and colorless for two to three minutes after the last addition of hydrogen peroxide. Ten milliliters of water is added through the condenser after the solution has cooled. The flask is removed from the condenser and 5% potassium permanganate is added, dropwise, with swirling until a pink color persists. Ten milliliters of 20% hydroxylamine hydrochloride solution is then added to the flask. The sample is filtered into a 50-ml. volumetric flask and the digestion flask is rinsed with several portions of water. The rinsings are added to the solution in the volumetric flask and the samples are made to volume with distilled water.

**Determination.**—Fifty milliliters of 0.25 *N* hydrochloric acid is placed in a 250-ml. separatory funnel. The proper aliquot of the digested solution is added and the contents of the funnel are mixed well. Twenty-five milliliters of dithizone solution (4 mg. per liter) is added and the contents of the funnel are shaken 60 times. A pledget of absorbent cotton is inserted into the outlet of the separatory funnel. The colored chloroform layer is then strained through the cotton into a sample cuvet and the absorbance of the solution is determined at 490 mμ with the Beckman DU spectrophotometer.

Aliquots are usually chosen so that the sample for determination will contain 10 to 25 mcg. mercury.

The amount of mercury in the digested solution is determined by comparison with a standard curve prepared according to the following procedure. Fifty milliliters of 0.25 *N* hydrochloric acid is placed in a 250-ml. separatory funnel and concentrated sulfuric acid equivalent to the amount of acid theoretically present in the aliquot taken is added. One milliliter of 20% hydroxylamine hydrochloride is added for each ten ml. of aliquot used. Finally, the proper amount of standard mercury solution is added and the solutions are mixed well. Twenty-five milliliters of dithizone solution is added and the absorbance is determined as described above. The standard curve follows Beer's law up to a concentration of 30 mcg. in the aliquot. For larger mercury concentrations a smaller aliquot must be chosen. If a large aliquot is used, enough water should be added to the standard preparations so that their volume will be approximately equal to the digest samples.

## DISCUSSION

When samples are low in moisture content and high in organic matter a 10-ml. quantity of concentrated sulfuric acid is usually necessary. If the samples are observed to be digesting slowly the second 5-ml. portion of acid is added through the condenser. Polley and Miller (4) list a number of different types of material and the variations in conditions of the digestion procedure necessary for each.

The size of the sample chosen may be varied according to mercury content. If larger samples are used the amount of acid must be increased.

Additions of hydrogen peroxide must be made very carefully to prevent a vigorous reaction. Expulsion of gas from the condenser is to be avoided. Great care is also necessary when heating the samples over the open flame, and should be only vigorous enough to cause the reaction to proceed. Excessively high and prolonged heating was found to reduce greatly the amount of mercury recovered from the samples.

The nature of the samples determines the filtration treatment necessary for the digests. Samples which are high in fat content may be chilled prior to filtration to solidify the fats. Samples with no insoluble residue need not be filtered. Filtrations are best accomplished through a fine-grade sintered-glass filter utilizing reduced pressure.

Polley and Miller recommended addition of sodium chloride to the samples after digestion and during the determination procedure to prevent adsorption of mercury onto the glass walls of containers and onto silica of soil samples. Under conditions of this experiment no adsorption of mercury could be detected, even though the samples were allowed to remain in the separatory funnels for periods up to thirty-six hours. Thus, further addition of chloride ion to the samples was unnecessary.

The color of the mercury-dithizone complex was stable up to one and one-half hours when

TABLE I.—RECOVERY OF KNOWN AMOUNTS OF MERCURY FROM SAMPLES (MERCURY ADDED AS MERCURIC CHLORIDE)

| Material | No. of Samples | Mercury Added, mcg. | Mercury Found, mcg. (Mean) | Max. Deviation, mcg. |
|---|---|---|---|---|
| Beef muscle | 7[a] | 100 | 98.7 ± 2.0[b] | −5.0 |
| Beef muscle | 7 | 10[c] | 9.7 ± 0.2 | −0.5 |
| | | | 9.7 ± 0.1 | −0.4 |
| Rhubarb root | 7 | 100 | 101.4 ± 0.9 | +3.0 |
| Soil | 7[d] | 100 | 99.9 ± 0.8 | +1.0 |

[a] For each material, an eighth sample containing no added mercury was carried through the procedure. Only in the case of the one fifth aliquot of the 10 mcg. beef muscle sample was mercury (1.0 mcg.) found in these samples.

[b] The standard deviation of the mean was calculated by the formula $\sigma = \sqrt{\frac{1}{n-1}\Sigma d^2}$.

[c] The first value was obtained using a one fifth aliquot of the sample digest. The second value was obtained using a one half aliquot.

[d] The samples were prepared with soil collected in one area.

TABLE II.—RECOVERY OF KNOWN AMOUNTS OF ORGANIC MERCURIAL

| Mercurial | No. of Samples | Mercury Added, mcg. | Mercury Found, mcg. (Mean) | Max. Deviation, mcg. |
|---|---|---|---|---|
| Di-*p*-tolylmercury | 4 | 100 | 100.8 ± 0.8 | +1.5 |
| Sodium ethylmercurithiosalicylate | 4 | 100 | 100.6 ± 1.3 | +2.5 |

stored in normal lighting conditions of the laboratory. No study was made on the effect of strong sunlight or artificial light. Strong light is probably best avoided however.

The effect of various metals which might possibly interfere in the determination of mercury was investigated. One milligram quantities of iron, nickel, lead, copper, zinc, cadmium, manganese, and bismuth were found to have no effect on the assay.

Although the procedure was developed primarily for use with animal tissue, the applicability of the method to certain mineral and vegetable materials was also tested. The results are summarized in Table I.

The procedure was tested to determine if it would be sensitive to organic mercurials as well as inorganic mercury. The results are summarized in Table II.

The accuracy of the experiment using the Beckman DU spectrophotometer seems to be comparable to that reported by Polley and Miller (4) i. e., 1 mcg. or 5%, whichever is greater. The overall time required for the whole procedure is approximately five hours depending upon the type of sample, the number of samples, and the experience of the operator. Since the procedure is less complicated and requires less manipulation of the samples than many other methods, there is less chance for loss of small amounts of sample and the consequent errors involved.

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# Color Stability of Tablet Formulations I*

## Measurement of Changes in Individual Tablet Reflectance

By TIBOR URBANYI, CHARLES J. SWARTZ, and LEON LACHMAN

**A holder which permits the centering and leveling of individual tablets in the reflectance attachment of the Beckman model DU spectrophotometer is described. By this adaptation, it is possible to measure the reflected light from the upper or lower surface of a tablet with a precision of two per cent. This method of measurement has proved useful in assigning reproducible numerical values for the light reflected from tablets.**

A PROMINENT CONSIDERATION in the stability evaluation of compressed tablets is the establishment of a tablet formulation which exhibits minimum alterations in color with storage. Although this may be desirable, it is a recognized fact that many tablet dosage forms showed change in color with time. Despite this, there are apparently no reports in the literature concerning the measurement of changes in appearance of tablets. An important reason for this situation may be the unavailability of suitable analytical techniques for measurement of color changes in individual tablets. It was, therefore, the purpose of this investigation to develop an analytical technique to accomplish the measurement of the aforementioned property.

Methods available for the standardization and evaluation of the appearance of tablets can be classified into two catagories, visual and instrumental. Visual estimation of tablet color is accomplished by comparison with standard tablets of the desired color or comparison with artificial standards such as color chips or charts. Comparison with standard tablets is unsatisfactory due to the possibility of subtle changes in the standard with time, while with color chips or standard reference charts of color, it is almost impossible to obtain these items to match the tablets under study.

Although the human eye is superior to instruments in measuring small hue and chroma differences, spectrophotometers are capable of more precise detection of small value differences (1). In order to study and predict shelf life of tablets from an appearance standpoint, an instrumental method capable of defining color intensity in reliable, reproducible values was indicated. The presently used instruments such as the "Color Eye" require relatively large samples which may be advantageous for batch control. However, because of the variations that may exist in color intensity between faces of single tablets and the resulting void spaces that form when several tablets are placed in the holder at one time, a method for individual tablets was desired. The method should define the appearance of the tablet in precise reproducible terms and be applicable to either the upper or lower face of the tablet. For this purpose a procedure was developed and a description, as well as representative data, obtained through the use of this method are presented.

* Received August 21, 1959, from the Research Department, CIBA Pharmaceutical Products, Inc., Summit, N. J.
Presented to the Scientific Section, A. PH. A. Cincinnati meeting, August 1959.

### EXPERIMENTAL

**Description of Tablet Holder.**—Since practically all analytical laboratories are equipped with highly accurate and precise spectrophotometers which can be used for the determinations of the reflectance of powders, it was decided to utilize the Beckman model DU spectrophotometer and its reflectance attachment for the measurement of the reflectance of individual tablets. In order to accomplish this, a tablet holder was designed to permit accurate, reproducible centering and leveling of the individual tablet in the reflectance attachment. Such a holder is shown in Fig. 1. The holder is made of oxidized steel with dimension *A* being the diameter of the tablet.

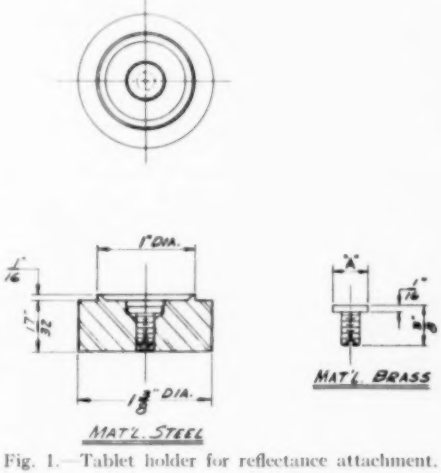


Fig. 1.—Tablet holder for reflectance attachment.

With minor modifications the holder can be adapted to other spectrophotometers.

**Procedure for Measurement.**—The tablet to be measured is placed in the tablet holder and the leveling screw of the holder is adjusted so that the top edge of the tablet is made level with the holder. The holder is mounted in the front compartment of the reflectance attachment, and a National Bureau of Standards Vitrolite plate of known reflectance is placed in the back compartment. The diffuse reflectance is measured in accordance with standard techniques. The reflectance can be measured in absorbance or per cent transmission at the desired wavelength.

## RESULTS AND DISCUSSION

A single tablet colored with FD&C Blue No. 1 was measured at its absorption maximum, 640 mμ, for a total of ten times, repositioning the tablet for each measurement of the upper and lower surface. The mean absorbance of the upper surface was 0.428 and the average deviation ±0.001, while the value for the lower face was 0.438 and the average deviation ±0.001 as represented in Table I. In a subsequent experiment ten different tablets from a single batch colored with FD&C Violet No. 1 were measured at their absorption maximum of 590 mμ. The mean absorbance at the upper face was 0.410 and the average deviation ±0.002, whereas the value for the lower face was 0.420 and the average deviation ±0.002 as shown in Table II. It is evident from the results presented in Tables I and II that excellent precision is obtained in the utilization of this technique for individual tablet measurement.

The diffuse reflectance can be measured at any suitable wavelength. In the case of tablets colored with a single dye, measurement at the absorption maximum of the dye is usually satisfactory. The variation of the reflectance of FD&C Violet No. 1 with wavelength is illustrated in Fig. 2.

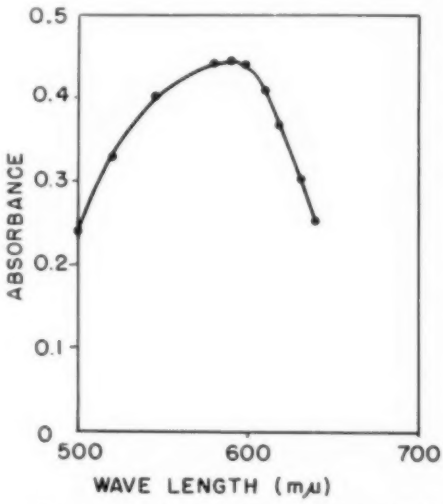


Fig. 2.—A plot showing the variation of absorbance with wavelength for tablets colored with FD&C Violet No. 1.

TABLE I.—ABSORBANCE AT 640 mμ OF SINGLE TABLET COLORED WITH FD&C BLUE NO. 1

| Absorbance Upper Face | Absorbance Lower Face |
|---|---|
| 0.427 | 0.437 |
| 0.427 | 0.437 |
| 0.428 | 0.438 |
| 0.428 | 0.438 |
| 0.428 | 0.438 |
| 0.428 | 0.438 |
| 0.428 | 0.438 |
| 0.428 | 0.439 |
| 0.429 | 0.439 |
| 0.430 | 0.439 |

TABLE II.—ABSORBANCE AT 590 mμ OF TEN TABLETS COLORED WITH FD&C VIOLET NO. 1

| Tablet | Absorbance Upper Face | Absorbance Lower Face |
|---|---|---|
| 1 | 0.408 | 0.417 |
| 2 | 0.408 | 0.418 |
| 3 | 0.409 | 0.419 |
| 4 | 0.409 | 0.420 |
| 5 | 0.410 | 0.420 |
| 6 | 0.410 | 0.420 |
| 7 | 0.411 | 0.421 |
| 8 | 0.411 | 0.421 |
| 9 | 0.411 | 0.421 |
| 10 | 0.412 | 0.422 |

TABLE III.—TRISTIMULUS POINTS ON FIVE COLORED TABLETS

| Wavelength, mμ | FD&C Violet No. 1 | FD&C Green No. 3 | FD&C Blue No. 1 | FD&C Red No. 1 | D&C Yellow No. 10 |
|---|---|---|---|---|---|
| 640 | 0.226 | 0.436 | 0.432 | 0.036 | 0.046 |
| 546 | 0.367 | 0.200 | 0.166 | 0.230 | 0.055 |
| 436 | 0.091 | 0.196 | 0.100 | 0.161 | 0.260 |

For tablets containing more than one dye or an excipient which may fade or darken, it may be preferable to measure at the tristimulus points (normally 436, 546, and 640 mμ) or in the yellow, blue, and red regions.

Table III lists the absorbances obtained at the tristimulus points for five different colored tablets. When utilizing the tristimulus technique, it is desirable to plot the data on triangular graph paper in order to obtain a better visualization of the changes in hue that result.

This procedure has been found useful in the determination of the color stability of tablets containing several different certified dyes and is reported in a subsequent paper of this series (2).

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# Color Stability of Tablet Formulations II*

## Influence of Light Intensity on the Fading of Several Water-Soluble Dyes

By LEON LACHMAN, CHARLES J. SWARTZ, TIBOR URBANYI, and JACK COOPER

**The photosensitivity of a number of commonly used, certified water-soluble dyes employed in tablets has been investigated. Exposures were made under both normal and exaggerated light intensities. In both cases the spectral character of the radiation was similar. The dyes evaluated included FD&C Red No. 1, FD&C Red No. 3, D&C Green No. 5, FD&C Green No. 3, FD&C Blue No. 2, FD&C Blue No. 1, FD&C Yellow No. 5, D&C Yellow No. 10, D&C Orange No. 3, and FD&C Violet No. 1. Apparent rates of fading for the several dyes were determined. The relationship between fading and dye structure as well as fading and light intensity is discussed. The extent of dye fading beneath the surface of the tablet was also measured.**

THE USE OF certified water-soluble dyes for imparting color to tablets is widespread in the pharmaceutical industry. The photosensitivity of these dyes, when incorporated into tablet formulations, represents a formidable problem. Although this is a generally recognized difficulty, there exists a conspicuous lack of information in the pharmaceutical literature concerning the influence of light on color fastness. This lack of data can be ascribed to several factors among which are (*a*) the unavailability of a suitable exaggerated-light stability cabinet, (*b*) the unavailability of appropriate analytical techniques for measurement of color changes at the surface of tablets, (*c*) the common practice of discontinuing the use of a specific color when found unsuitable in a given formulation without determining the nature of the objectionable characteristic, and (*d*) the treatment of such data as confidential within the particular company.

Because of the practical significance of this problem, a vast store of empirical knowledge on the subject has been accumulated in the pharmaceutical as well as other industries. Although pharmaceutical references are sparse, numerous reports exist on the nature of fading reactions in the textile, dye, and plastic industries. In summarizing this information certain aspects predominate. Depending upon their chemical constitution, dyes behave in many different ways under the influence of light. They may fluoresce (1), be oxidized or reduced, either reversibly or irreversibly (2, 3), or may sensitize the reaction of other components of the irradiated system (4, 5 ). Furthermore, the quality and intensity of light as well as the continuous or intermittent nature of exposure strongly affect the fading reaction (6).

In light of recent Food and Drug Administration rulings regarding the decertification of many commonly used colorants, maximum utilization of all approved dyes now seems imperative. In order to accomplish this it is essential to ascertain the stability characteristics of the remaining certified dyes.

A study was therefore initiated to determine the light stability of ten certified water-soluble colors when used in tablet dosage forms. The purpose of this study was not the elucidation of specific fading mechanisms of the several colors but instead the determination of the relative stability of the colors under normal and exaggerated illumination. The basic tablet formulation used for this investigation was composed of relatively inert materials. The fading at the surface of the tablet was determined following exposure to so-called "normal" and "exaggerated" light intensities. Apparent rates of fading for the several colored tablets following exposure were calculated. The relationship of degree of fading at various depths from the tablet surface was measured for representative tablets. The possibility of the existence of a general relationship between the extent of fading under ordinary and accelerated illumination is discussed.

### EXPERIMENTAL

**Materials Used.**—FD&C Red No. 1, FD&C Red No. 3, D&C Green No. 5, FD&C Green No. 3, FD&C Blue No. 2, FD&C Blue No. 1, FD&C Yellow No. 5, D&C Yellow No. 10, D&C Orange No. 3, and FD&C Violet No. 1.

**Equipment.**—Light stability cabinets exhibiting intensified and normal room illumination as described in a previous publication (7), Gossen Tri Lux foot candle light meter, Stokes model F tablet

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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

press, Beckman spectrophotometer model DU with reflectance attachment, die attachment for centering and leveling tablets in reflectance units as described in previous paper of this series (8), precision rotary microtome, and Beckman aquameter.

**Preparation of Tablets.**—Tablets containing the various water-soluble certified dyes were prepared according to the following formula:

| | |
|---|---|
| Calcium sulfate, dihydrate | 96.99% |
| Tragacanth U. S. P. | 2.00% |
| Magnesium stearate, U. S. P. | 1.00% |
| Dye | 0.01% |
| Ethyl alcohol 50% | q. s. |

A granulation of the above formula was prepared in accordance with commonly-employed tableting procedures. The dye was dispersed by dissolving it in the 50% alcohol which was used as a granulating agent. Tablets weighing 700 mg. were compressed using $^{16}/_{32}$ inch flat punches, uppers bisected. The tablets were all compressed to a hardness of approximately 7 Kg/in.$^2$ and a thickness of 3 mm. on a Stokes model F tablet press. The moisture content of the dried granulations was kept consistently below 0.5%.

**Storage of Samples in the Light Stability Cabinets.**—Adequate samples of the several differently colored tablets were put into three-inch uncovered clear-glass Petri dishes which were then placed into the normal and intensified light cabinets. The intensity of the light incident on the tablets in the respective cabinets was kept at a constant level through measurement of the light intensity with a Gossen Tri Lux meter at regular time intervals. The light intensities falling on the tablets in the normal and exaggerated illumination cabinets were 45 and 550 foot candles, respectively. Tablet samples were withdrawn from the cabinets at designated time intervals for reflectance measurements.

**Measurement of Tablet Reflectance.**—The tablets removed from the light stability cabinets were measured for fading of the color at the tablet surface by the use of a Beckman model DU spectrophotometer with a modified Beckman reflectance unit attachment to permit its use for single tablets. The equipment modifications and method of measurement are described in detail in a previous publication (8). Reflectance measurements were made at 640 mμ for FD&C Blue No. 1, at 500 mμ for FD&C Red No. 1, at 540 mμ for FD&C Red No. 3, at 620 mμ for FD&C Green No. 3, at 630 mμ for D&C Green No. 5, at 420 mμ for D&C Yellow No. 1, at 430 mμ for FD&C Yellow No. 5, at 610 mμ for FD&C Blue No. 2, at 590 mμ for FD&C Violet No. 1, and at 500 mμ for D&C Orange No. 3.

**Extent of Fading Beneath Surface of Tablet.**—In order to determine the degree of fading at various distances from the surface of the tablet individual tablets were sliced to definite thicknesses with a microtome. Each tablet was placed in a die which was subsequently leveled in the microtome to permit uniform vertical cuts of the flat surface of the tablet. Reflectance measurements were performed for the several colored tablets at distances up to 300 μ from the surface.

**Determination of Moisture Content of Tablets.**—Water contained in the tablets was measured by Karl Fischer titration, the end point being determined electrometrically utilizing the Beckman aquameter. Sample tablets were crushed in a mortar and weighed aliquots removed for dispersion in methanol. This dispersion, under constant stirring, was then titrated with Karl Fischer reagent.

## RESULTS AND DISCUSSION

The photosensitivity of the several dyes used in the tablets studied was determined by exposure to normal and intensified illumination.

Representative plots showing a decrease in the spectral absorbance of the surface of the tablets colored with FD&C Blue No. 1, FD&C Yellow No. 5, and FD&C Blue No. 2 are presented in Figs. 1, 2, and 3. It can be seen from these curves that a substantial variance in the degree of color fading exists, depending upon whether the tablets are exposed under normal or exaggerated illumination. In addition, the rate of fading of the surface color of the tablets is not the same for the several dyes studied, but varies according to the individual color used. An interesting fact brought out by these curves is the change that results in the absorption maximum as the fading progresses. A flattening as well as a hypsochromic shift of the absorption maximum occurs. The magnitude of this effect is greatest for FD&C Blue No. 1 and least for FD&C Blue No. 2, with FD&C Yellow No. 5 falling in between.

Representative plots indicating the rate of fading of dye from the surface of the tablets are given in Figs. 4 and 5. It is readily evident from the linearity of the plots that the fading follows an apparent first-order reaction. These graphs further show the presence of more than one slope for the plots of log absorbance *vs.* time for most of the samples studied. In all cases, the initial rate of fading was greater than that obtained from subsequent portions of the fading curve. Baxter, *et al.* (9), in their studies of the fastness of dyes in textiles also found fading to

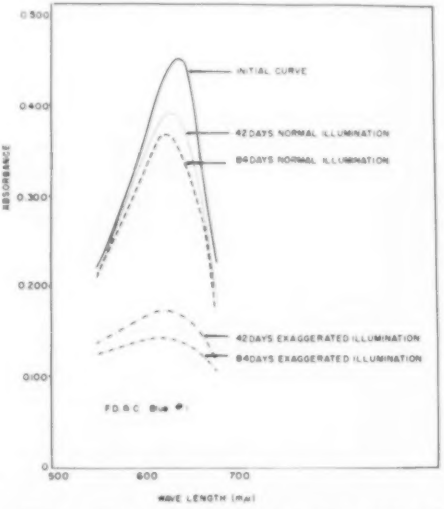


Fig. 1.—Plots of the visible absorption spectra of FD&C Blue No. 1 after different intervals of storage under normal and exaggerated illumination.

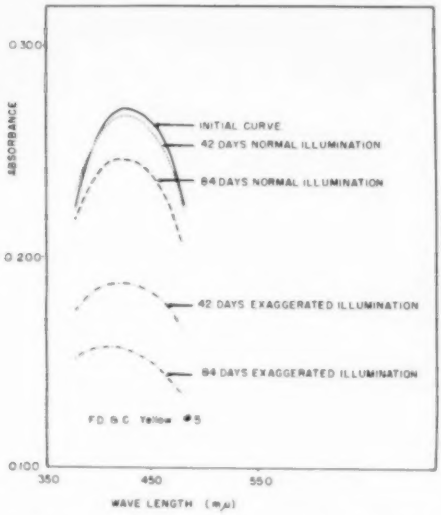


Fig. 2.—Plots of the visible absorption spectra of FD&C Yellow No. 5 after different intervals of storage under normal and exaggerated illumination.

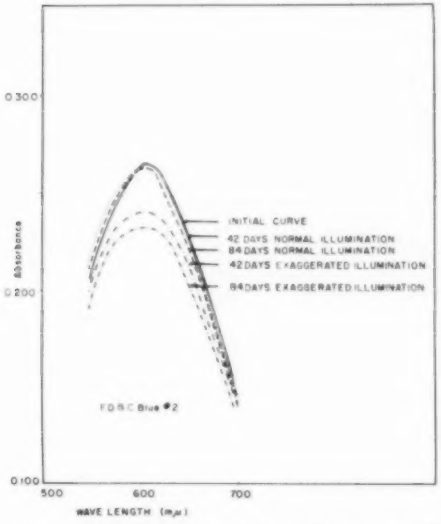


Fig. 3.—Plots of the visible absorption spectra of FD&C Blue No. 2 after different intervals of storage under normal and exaggerated illumination.

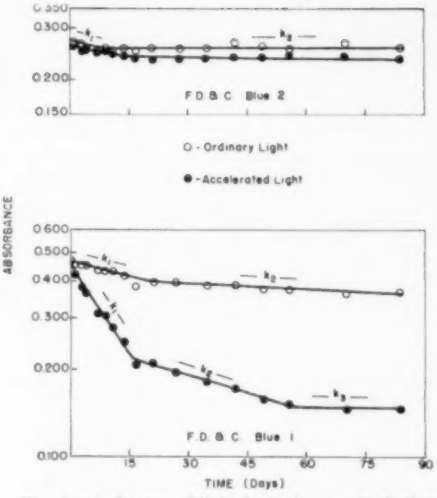


Fig. 4.—Influence of light intensity on the fading of the surfaces of tablets colored with FD&C Blue No. 2 and FD&C Blue No. 1.

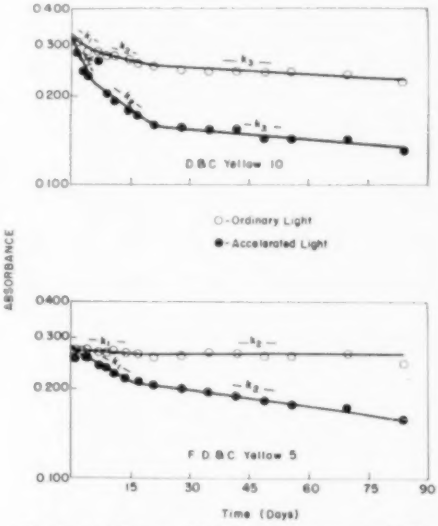


Fig. 5.—Influence of light intensity on the fading of the surface of tablets colored with D&C Yellow No. 10 and FD&C Yellow No. 5.

be much more rapid during the earlier stages of irradiation.

The changes in reaction rates that are observed as the duration of irradiation is increased do not necessarily indicate that the fading mechanism consists of a series of consecutive reactions of different rates. Instead, they are most likely due to several contributing factors, among which can be (*a*) alterations in the quantity of light reflected and absorbed at the surface of the tablet as fading proceeds, (*b*) subsequent changes in the amount of energy available to bring molecules to the excited state, (*c*) possible absorption of a certain amount of energy by the decomposition products, (*d*) porous nature of the tablets, (*e*) influence of the substrates on the photoreaction, (*f*) the possibility of reactions between dye and its decomposition products, (*g*) secondary reactions involving the dye and atmosphere.

Because it was felt that one or more of the above

factors probably contribute to the changes in the rates observed in the plots of log absorbance *vs.* time it was decided to utilize the initial fading rate as most accurately representing the photodegradation of the dye at the tablet surface. This postulation seems to be supported by Desai and Giles (3) and Atherton and Seltzer (10) in their studies on dye fading. Desai and Giles indicated that the initial reaction is representative of an attack on one or other of the centers of the molecule which are responsible for color. Atherton and Seltzer based their experiments on measurements made over the initial stages of reaction in order to minimize the effect of reactions between the dye and its decomposition products.

The apparent first-order reaction rates for the disappearance of color from the surface of the tablets upon exposure to light were obtained from the slopes of the plots of log absorbance *vs.* time according to the equation, $\log A_t = -[k/2.303]\,t + \text{constant}$, where $-k/2.303$ is the slope of the plots. The rate constants ($k$) evaluated in this manner are presented in Table I. Although the rate constants for the different slopes of each curve are given, only the initial rate, $k_1$, will be used in subsequent discussions for reasons previously mentioned.

It is evident from the data presented in Table I that the exaggeration produced by the intensified light source on the rate of fading of the several differently colored tablets is dependent upon the dye used and does not follow any particular pattern. The acceleration in fading rate caused by the exaggerated illumination ranged from 1.5-fold for FD&C Blue No. 2 to 240-fold for D&C Green No. 5, when compared with normal illumination fading.

The contribution of structural configuration of the dye molecule to its photosensitivity is a generally accepted fact in the field of dye chemistry (11). In addition, it is somewhat hazardous to postulate a general relationship between color fastness and structural classification of the dye, i. e., triphenylmethane, azo, indigoid, etc., since it has been shown by Desai and Vaidya (12) that dyes of the same chemical classification vary in fastness to light.

In this study, the dyes used in the tablets exposed to normal illumination exhibit the following decreasing order of light stability: D&C Green No. 5 > D&C Orange No. 3 > FD&C Yellow No. 5 > FD&C Blue No. 2 > FD&C Green No. 3 > FD&C Blue No. 1 > FD&C Violet No. 1 > FD&C Red No. 1 > D&C Yellow No. 10 > FD&C Red No. 3. On the other hand, the tablets exposed to the exaggerated illumination exhibit the following decreasing order of light fastness: FD&C Blue No. 2 > FD&C Yellow No. 5 > D&C Orange No. 3 > FD&C Green No. 3 > FD&C Blue No. 1 > D&C Green No. 5 > FD&C Red No. 1 > D&C Yellow No. 10 > FD&C Violet No. 1 > FD&C Red No. 3. It is thus apparent that several of the dyes under exaggerated illumination do not possess the same order of relative light fastness as exhibited under normal illumination.

It has been reported by Taylor and Pracejus (6) in their study on the fading of colored plastics and textiles, that due to the porous mesh nature of textiles, depth penetration of all radiations is permitted, subsequently causing color fading at appreciable depths from the surface. Since compressed tablets vary in porosity as a result of the particle size of the granulation as well as the compression force used to prepare them, it is believed that depth fading should also occur in tablets. In order to ascertain this, tablets colored with FD&C Violet No. 1 and exposed to the normal and exaggerated illumination for a period of eighty-four days were microtomed to different depths from the surface. Reflectance measurements were then performed on these new surfaces and the data are presented in Fig. 6.

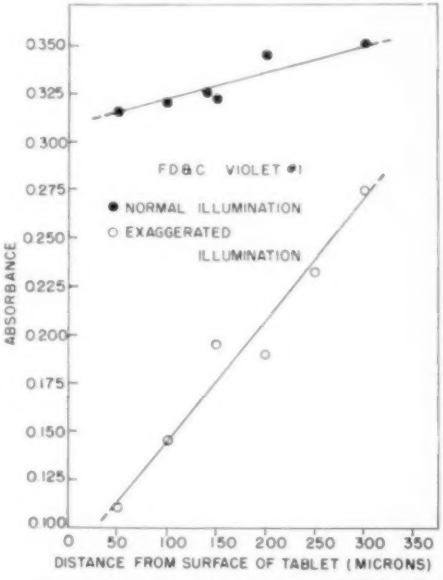


Fig. 6.—A plot showing the extent of fading beneath the surface of tablets colored with FD&C Violet No. 1.

TABLE I.—RATE CONSTANTS FOR THE FADING OF COLORS IN COMPRESSED TABLETS IN DAYS$^{-1}$

| Color | Ordinary Lighting | | | Accelerated Lighting | | |
|---|---|---|---|---|---|---|
| | $k_1 \times 10^3$ | $k_2 \times 10^3$ | $k_3 \times 10^3$ | $k_1 \times 10^3$ | $k_2 \times 10^3$ | $k_3 \times 10^3$ |
| FD&C Red No. 1 | 15.8 | 5.11 | 1.09 | 69.0 | 19.5 | 4.4 |
| FD&C Red No. 3 | 71.2 | 22.2 | 5.9 | 264.0 | 25.5 | 9.2 |
| FD&C Blue No. 1 | 5.48 | 1.63 | ... | 50.6 | 9.4 | ... |
| FD&C Blue No. 2 | 3.45 | ... | ... | 5.75 | 0.15 | ... |
| FD&C Green No. 3 | 4.55 | 0.75 | ... | 29.2 | 10.9 | 4.98 |
| D&C Green No. 5 | 0.25 | ... | ... | 59.8 | 20.4 | 1.88 |
| FD&C Yellow No. 5 | 2.04 | 0.38 | ... | 16.3 | 4.78 | ... |
| D&C Yellow No. 10 | 16.4 | 8.70 | 1.22 | 72.1 | 22.1 | 3.29 |
| FD&C Violet No. 1 | 14.6 | 6.13 | 2.26 | 78.0 | 35.6 | 8.36 |
| D&C Orange No. 3 | 0.25 | ... | ... | 20.7 | 2.88 | ... |

It is readily evident from the resulting plots that a substantial amount of fading takes place beneath the surface of the tablet. Furthermore it is interesting to note that by plotting absorbance *vs.* distance from the surface, straight line plots are obtained for the tablets colored with FD&C Violet No. 1 exposed to normal and exaggerated illumination as evidenced in Fig. 6. Because the differently colored tablets used in this study were prepared from granulations of the same particle size distribution as well as being compressed to the same hardness, they should all exhibit approximately the same porosity. Accordingly, they should also exhibit color fading beneath the surface. Two additional tablets, tablets colored with FD&C Blue No. 1 and D&C Green No. 3, were tested similarly to FD&C Violet No. 1 in order to determine the correctness of this assumption. These two tablets also exhibited significant fading of color beneath the tablet surface.

Inasmuch as fading of colored tablets does not result in a uniform reduction in dye concentration throughout the entire tablet but rather is, to a large extent, a surface phenomenon, it is difficult to correlate extent of fading with the destruction of a certain proportion of the dye initially present in the tablet. Therefore, when the reflectance at the surface of the tablet shows that the dye has faded 50% it does not correspond to 50% total dye destroyed.

The tablet excipients were carefully chosen for this study so that they would not exhibit any significant substrate effect on the fading rate. In addition, the moisture content of the tablets was kept below 0.5% in order to prevent exaggeration of the fading reaction.

Although it may be desirable to use a monochromatic light source for the measurement of dye photosensitivity, a polychromatic source was used in this study in order to simulate practical storage conditions.

## SUMMARY AND CONCLUSIONS

The photosensitivity of ten water-soluble certified dyes in tablets exposed to normal and exaggerated illumination is evaluated. From the results of this study the following conclusions can be drawn:

1. The absorption curves presented for FD&D Blue No. 1, FD&C Yellow No. 5, and FD&C Blue No. 2 show a hypsochromic shift as well as a flattening of the absorption maximum in the visible spectrum as fading progresses.

2. The apparent degradation rates for the various certified dyes studied are presented and appear to follow a first-order reaction.

3. The fading curves for the dyes investigated show that the rate is most rapid during the initial stage of irradiation.

4. The exaggeration of color fading produced by the intensified illumination does not follow a particular pattern for the dyes studied. The potentiation of the fading rate varies from 1.5- to 240-fold, depending upon the dye studied.

5. Fading beneath the surface of the tablet has been found in the several colored tablets studied. This depth fading can be ascribed to the inherent porosity of compressed tablets.

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# Serotonin-Induced Apnea*

By D. C. KROEGER and L. J. LUCCO†

**Studies have been conducted on a number of species of animals to determine the respective role of peripheral and central actions of serotonin which causes apnea. In dogs, three respiratory events are obtained with i. v. injections of serotonin; transient gasping and a slower developing hyperpnea which is interrupted in a majority of the dogs by apnea. Both forms of respiratory stimulation are sensitive to procaine endoanesthesia, gasping being selectively blocked by carotid sinus denervation. Hypercapemia resulting from apnea and bronchoconstriction play a role in the hyperpnea. Apnea in the dog is of a different origin than that in the cat. Preventing bronchoconstriction reduces the occurrence of apnea in the dog. Evidence is presented for a central (medullary) inhibition of reflex gasping by serotonin.**

SPECIES DIFFERENCES to the effects of serotonin, 5-hydroxytryptamine (5-HT) are well known (1). Cats and dogs show opposite respiratory responses to intravenous injections of 5-HT. In cats the usual response is apnea which is followed by hyperpnea, whereas in the dog, apnea is frequently seen to interrupt an existing hyperpnea.

Currently many investigators and reviewers attribute the apnea to a reflex originating from the stimulation of receptors lying in the cardiopulmonary circulation circuit (2–6) and the hyperpnea to a reflex arising from stimulation of receptors in the aorto-carotid baro- and chemoceptive areas (7–9). The situation is much confused, however, for not only does 5-HT stimulate peripheral receptors (10), but exerts a direct effect upon ganglionic transmission (11), smooth muscle (12), and central nervous system (13), and also releases histamine into the general circulation (14).

A survey of the literature suggests, however, that some of the current confusion regarding the respiratory actions of 5-HT lies in the fact that certain phenomena involved with respiration have not been considered. The terms respiratory stimulation, hyperpnea, and apnea have been used with little clarification as to whether these consisted of changes in rate, amplitude, duration and/or even changes in the type of respiration. Thus, the purpose of this study is to define certain respiratory characteristics of 5-HT, to present evidence for some possible mechanisms of actions, and to review the pertinent literature in view of these findings.

* Received August 21, 1959, from the Pharmacology Laboratories, Dental Branch, University of Texas, Houston 25.
Data concerning blood gas analyses is abstracted from a thesis submitted to the Graduate School of the University of Houston by L. J. Lucco in partial fulfillment of the requirements for the Master of Science degree.
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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

## EXPERIMENTAL

**Methods.**—Forty-five dogs weighing 8–16 Kg.; 15 cats, 2–3 Kg.; two rhesus monkeys, 2–3 Kg.; and three chickens weighing 0.5 Kg. were used in this study. Various anesthetic mixtures were used including i. v. pentobarbital, 30 mg./Kg.; pentobarbital-morphine, 10 mg. each/Kg.; and chloralose, 75–90 mg./Kg.

For routine experiments, blood pressure was recorded from the femoral artery with a Statham or E+M blood pressure transducer. Respiration in dogs and certain of the other animals was recorded using an intrathoracic trochar attached to a Statham transducer, otherwise, chest movements were recorded using an E+M stethograph. After tracheal cannulation, respiratory exchange was measured with a Benedict-Roth spirometer and continuous blood oxygen levels monitored with an E+M flow-through oximeter connected to the carotid circulation. Carotid and jugular blood samples were simultaneously obtained anaerobically and analyzed for blood gases by the Van Slyke method. The carotid bifurcations were exposed and the carotid sinus and carotid body nerves isolated and identified using the protocol outlined by Neil, *et al.* (15). The majority of injections were administered rapidly into the femoral vein with a saline wash, other injections were made into the carotids and jugulars. Sodium mannuronate, 10 mg./Kg., was used to prevent clotting.

Special procedures for six cats consisted of bilateral isolation of the carotid sinus and body with their nerves. The floor of the fourth ventricle was exposed by removal of the cerebellum and evoked potentials recorded using insulated nichrome electrodes RC coupled to a dual-beam oscilloscope. Electrode placement and microinjection of drugs into the medulla were made using a Lab-Tronic stereotaxic apparatus. Carotid blood pressure was recorded with a Hathaway transducer and written out on the Physiograph[1] as was all other transduced data. Blood pressure was maintained by slow i. v. infusion of norepinephrine and body temperature held near normal with a heating pad.

Doses of 5-HT are listed as mcg. 5-HT base/Kg. of body weight. Two different samples of serotonin creatinine sulfate were available for this study (Abbott Laboratories and California Foundation

[1] Manufactured by the E+M Instrument Company, Houston, Tex.

for Biochemical Research); however, no difference in activity was noted. Except where noted, at least fifteen minutes was allowed between repeat injections of 5-HT. Other drugs used in this study were procaine hydrochloride, bufotenine monooxylate (California Foundation for Biochemical Research), lysergic acid diethylamide (Sandoz, Delysid), reserpine phosphate (Ciba), and (α-ethyl-3-piperidyl)-phenylcyclopentyl glycolate (Lakeside J.B. 329).

**Results.**—Rapid injection of doses of 5-HT from 1 to 100 mcg./Kg. were given into the femoral vein of dogs. The latency for the initial sign of a respiratory change was 6.04 ± 1.8 seconds. Bradycardia was usually seen simultaneously with the occurrence of the respiratory change. Immediately after the initial hyperventilation, four response patterns were observed (in the order of decreasing occurrence): a period of respiratory arrest; a decrease in the respiratory rate of amplitude with regard to the control value; continuation of the increased respiratory activity, only of less intensity than that of the initial response; a period in which the respiratory rate and amplitude rapidly returned to the control value. Generally the same response continued to be shown in the same dog with repeated injections.

The usual response obtained is shown in Fig. 1. With the injection of 30 mcg./Kg. of 5-HT there is a brief period averaging 12.1 ± 3.8 seconds of extreme respiratory effort, not only has the rate increased an average of 4.7 ± 1.6 times but there is an increase in the expiratory as well as the inspiratory air. Following this period of both tachypnea and hyperpnea there begins a period of respiratory arrest in the expiratory stage. Negative pressure in the thoracic cavity during the arrest period is slightly less than the control period, indicating a relaxation of the muscles about the thoracic cage. Following the period of complete arrest the respiratory muscles are again contracted to a much greater extent than normal but at a much slower rate than the control. Within a minute's time the rate has increased to slightly more than control and the amplitude of inspiration has decreased but is still approximately twice the control. The respiratory picture slowly declines both in amplitude and rate until it is back to control levels in five to ten minutes.

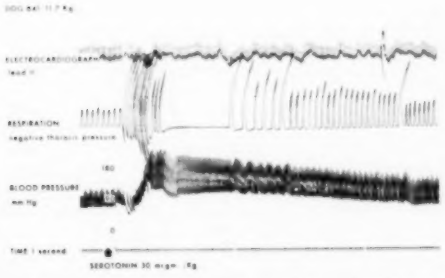


Fig. 1.—Dog 11.7 Kg., male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injection of 5-HT on electrocardiogram, respiration, and blood pressure in the intact anesthetized dog. Electrocardiograph; lead II, respiration; intrathoracic pressure, inspiration up. Blood pressure in mm. Hg. Time intervals are one second and one minute.

Figure 2 is a similar trace upon which we have indicated certain theoretical phenomena. The three phases represented here compare favorably with the three phases of 5-HT action described by Schneider and Rinehart (16). In this tracing there first appears inspiratory efforts which resemble and have been labeled as gasps. Shortly following the initial gasp there begins a secondary type of respiratory stimulation which involves not only an increase in the inspiratory effort but also forced expiration. Both of these events are suddenly terminated by respiratory arrest with the intrathoracic pressure again somewhat greater than during the control period. The inhibition of inspiration is terminated first but is shortly followed by the reappearance of a forced expiration. At this dose the hyperpnea, but not tachypnea, continues for an additional five minutes.

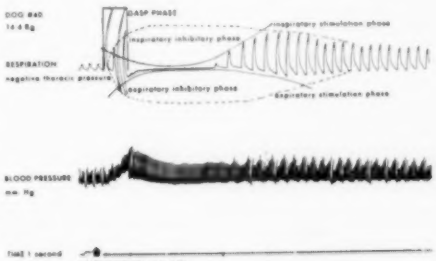


Fig. 2.—Dog 14.4 Kg., male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injection of 5-HT on respiration and blood pressure in the intact anesthetized dog. Respiration, intrathoracic pressure, inspiration up. Blood pressure in mm. Hg. Time intervals are one second and one minute. Three phases of the respiratory reaction are designated; phase 1 (gasp phase), phase 2 (combined inspiratory and expiratory excitatory phase), and phase 3 (combined inspiratory and expiratory inhibitory phase).

Another reaction which would influence the latter phase of respiratory stimulation is seen in Fig. 3. With the injection of 30 mcg./Kg. 5-HT, the increase in the depth and rate of respiration causes the oxygen saturation to increase briefly but it then begins to fall dramatically even though the respiration appears to be more than adequate. During the inhibitory phase there are a few aborted inspirations. The spirometer trace, however, indicates a lack of tidal movement in spite of rather rapid and excessive respiratory movements. The lack of gaseous exchange would appear largely due to bronchoconstriction rather than pulmonary vasoconstriction since the blood pressure tracing does not indicate an interference in cardiac output due to poor filling of the left heart. These findings confirm those of Konzett (17). Recent data by Borst, *et al.* (18), however, indicates a considerable pulmonary vasoconstriction by 5-HT and perhaps the lung by-pass via pulmonary A-V fistulas described by Sparks and Tombridge (19) might play a role. Reid and Rand (20), Comroe, *et al.* (2), and Brocklehurst (12) have demonstrated considerable bronchoconstriction.

A series of 12 dogs were studied with respect to blood gases following i. v. injection of 5-HT, 13

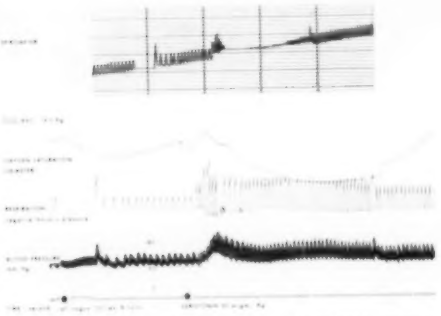

Fig. 3.—Dog 14.0 Kg., male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injection of 5-HT on respiration and blood pressure in the intact anesthetized dog. Top trace is tidal air recorded on a spirometer, vertical lines are one minute time intervals. Second trace is carotid artery oxygen saturation. Third trace is intrathoracic pressure, inspiration up. Spirometer trace is not enlarged to the same scale as Physiograph trace. Blood pressure in mm. Hg. Time intervals are one second and one minute. Note the loss of tidal air volumes which coincide with a fall in carotid artery oxygen saturation in spite of continued respiratory movements.

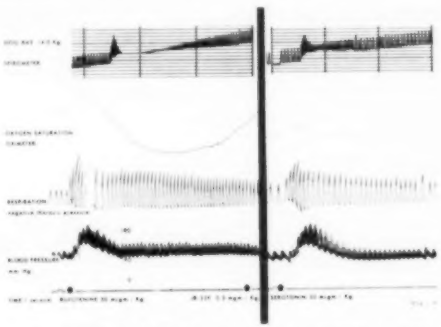

Fig. 4.—Dog 14.0 Kg., male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injections of bufotenine and 5-HT on respiration and blood pressure in the intact anesthetized dog. Both drugs were given before and after JB 329, one trace of each is presented. Similar reactions to both bufotenine and 5-HT were obtained. Top trace is the tidal air recorded on a spirometer, vertical lines are one minute intervals. Second trace is carotid artery oxygen saturation. Third trace is intrathoracic pressure; inspiration up. Lower trace is blood pressure in mm. Hg. Time intervals are one second and one minute. Note only partial reduction of tidal air volumes and lack of apnea following injection of JB 329.

mcg./Kg. Three doses of 5-HT were administered forty-five minutes apart and carotid and jugular blood samples collected just prior to the administration of 5-HT and immediately after termination of the initial respiratory stimulation. In this study at this dosage, only 4 of the 12 dogs showed apnea. A comparison of the mean oxygen and carbon dioxide contents of the samples pre- and postinjection showed a slight increase in the volume per cent of oxygen which was confirmed in the studies using the flow-through oximeter. Volume per cent of carbon dioxide showed a decrease. Due to individual variations, analysis of variance indicated little treatment significance. Table I is a summary of the blood gas analyses of the four animals showing apnea.

That this secondary respiratory stimulation is not entirely caused by hypoxia and/or hypercapemia is illustrated in Figs. 4 and 5. Figure 4 illustrates the action of both bufotenine, *N*-methyl-5-hydroxytryptamine and 5-HT which are nearly identical. Bufotenine is capable of causing a strong bronchoconstriction and a cessation of tidal movement of air. However, fifteen minutes after the injection of J. B. 329, 0.2 mcg./Kg., an anticholinergic compound, the bronchoconstriction is greatly reduced. This drug also blocks the apneic stage without influencing either the initial gasp or the secondary hyperpnea. Reserpine, 0.5 mg./Kg. and L. S. D. 25, 25 mcg./Kg., were also capable of blocking the bronchoconstriction but reserpine did not alter the respiratory events. This action of reserpine had previously been noticed by Schneider and Rinehart (16).

Figure 5 illustrates the progressive increase in the amount of bronchoconstriction with doses of 10, 20,

TABLE I.—OXYGEN AND CARBON DIOXIDE CONTENTS OF ARTERIAL AND VENOUS BLOOD IN DOGS BEFORE AND AFTER THE ADMINISTRATION OF SEROTONIN[a]

| | | | Arterial Blood | | | | | | Venous Blood | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Oxygen | | | Carbon Dioxide | | | Oxygen | | | Carbon Dioxide | | |
| Dog No. | Wt., Kg. | Inj. No. | Before Inj., Vol. % | After Inj., Vol. % | Change, % | Before Inj., Vol. % | After Inj., Vol. % | Change, % | Before Inj., Vol. % | After Inj., Vol. % | Change, % | Before Inj., Vol. % | After Inj., Vol. % | Change, % |
| 2 | 10.65 | 1[b] | 16.22 | 18.06 | 1.13 | 45.09 | 42.83 | −0.50 | 14.78 | 14.97 | 0.13 | 47.55 | 45.91 | −0.34 |
| | | 2[b] | 16.17 | 17.10 | 0.58 | 42.27 | 39.66 | −0.62 | 15.19 | 11.68 | −2.31 | 47.79 | 45.52 | −0.48 |
| | | 3[b] | 16.15 | 18.54 | 1.48 | 44.47 | 39.47 | −1.12 | 11.84 | 12.83 | 0.84 | 44.18 | 45.59 | 0.32 |
| 3 | 11.25 | 1 | 17.79 | 15.42 | −1.33 | 48.97 | 39.07 | −2.02 | 15.28 | 21.68 | 4.19 | 52.76 | 50.50 | −0.43 |
| | | 2 | 18.44 | 19.31 | 0.47 | 45.55 | 43.67 | −0.41 | 16.80 | 17.82 | 0.61 | 51.02 | 50.50 | −0.10 |
| | | 3[b] | 17.33 | 20.66 | 1.92 | 38.51 | 45.28 | 1.76 | 15.42 | 4.73 | −6.93 | 49.51 | 55.17 | 1.14 |
| 10 | 7.48 | 1[b] | 13.04 | 13.75 | 0.54 | 42.43 | 40.98 | −0.34 | 6.46 | 7.28 | 1.27 | 47.90 | 47.38 | −0.11 |
| | | 2[b] | 14.04 | 14.47 | 0.31 | 42.00 | 38.88 | −0.74 | 5.73 | 6.75 | 1.78 | 46.75 | 46.23 | −0.11 |
| | | 3[b] | 13.31 | 14.47 | 0.87 | 42.00 | 41.74 | −0.06 | 6.22 | 6.75 | 0.85 | 45.92 | 39.92 | −1.31 |
| 11 | 10.48 | 1 | 13.95 | 14.86 | 0.65 | 39.30 | 38.35 | −0.24 | 9.62 | 9.08 | −0.56 | 44.07 | 43.97 | −0.02 |
| | | 2[b] | 15.89 | 15.46 | −0.27 | 34.60 | 36.04 | 0.42 | 6.00 | 5.72 | −0.47 | 41.88 | 43.50 | 0.39 |
| | | 3[b] | 14.18 | 13.47 | −0.50 | 32.10 | 34.20 | 0.65 | 4.71 | 5.77 | 2.25 | 41.01 | 43.48 | 0.60 |

[a] The animals included in this table are only those that demonstrated a distinct apneic response following the initial hyperventilation elicited by the administration of serotonin base (13 mcg./Kg. i. v.). All percentage changes are positive unless noted.

[b] Injections in which apnea was observed.

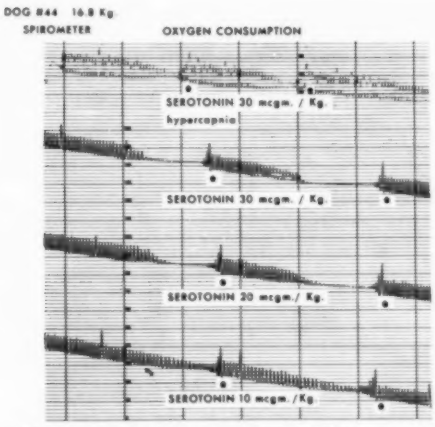


Fig. 5.—Dog 16.8 Kg., male. Anesthetic, pentobarbital-morphine. Effect of various intravenous doses of 5-HT on tidal air and oxygen consumption in the intact anesthetized dog. Spirometer traces read from right to left. Top trace illustrates the effect of increasing the carbon dioxide tension by removal of the soda-lime cannister.

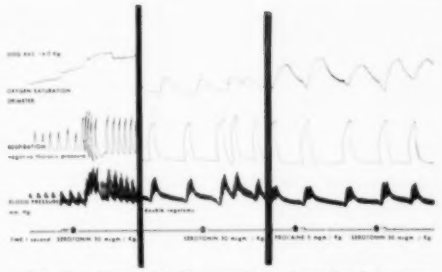

Fig. 6.—Dog 14.0 Kg., male. Anesthetic, pentobarbital-morphine. Effect of intravenous injections of 5-HT on carotid artery oxygen saturation, respiration; intrathoracic pressure and blood pressure in the intact and vagotomized dog. The tracings show the occurrence of a respiratory gasp in the vagotomized dog and blockage by intravenous procaine.

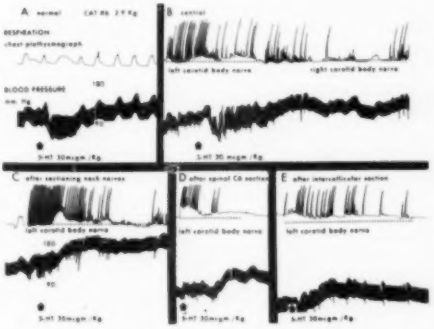


Fig. 7.—Cat 2.9 Kg., female. Anesthetic, chloralose. Effect of intravenous injections of 5-HT on the reflex gasping caused by intermittent tetanizing stimulation of the right or left carotid body nerve. Part A illustrates control responses to 5-HT. Part B illustrates inhibition of gasping by 5-HT in the intact cat. Part C illustrates the same response after sectioning of the vagi and the sympathetic chain. Part D shows a similar effect still present after spinal cord $C_8$ section. Part E shows this same effect after intercollicular sectioning of the brain stem. Dots appearing under respiration indicate frequency and duration of stimulation.

and 30 mcg./Kg. of 5-HT. Though these doses were repeated within three minutes there was no apparent desensitization or tachyphylaxis of this bronchoconstriction. The top tracing of Fig. 5 shows the lessening of the bronchoconstriction when the animal was made hypercapemic by removal of the soda-lime absorbent from the spirometer filled with 100% $O_2$.

Contrary to the report by Schneider and Yonkman (21), procaine 3 mg./Kg. is capable of blocking the initial reflex respiratory stimulation in dogs. With an injection of 5-HT, one to three minutes following the i. v. injection of procaine, the dog shows respiratory arrest or partial inhibition immediately without the respiratory stimulation.

Figure 6 illustrates the reflex gasp still obtained after double cervical vagotomy. Procaine HCl, 5 mg./Kg. i. v., however, abolishes this residual action of 5-HT, 30 mcg./Kg. Sectioning of the vagi abolishes the apneic response to small or moderate dosages of 5-HT; however, if the dose is increased up to 100 mcg. or more/Kg. i. v. in the vagotomized animal, another apneic response is obtained.

To investigate this action further a series of four cats with exposure of the medulla and the carotid bifurcation were employed. Decerebrate cats were made apneic by threshold tetanizing stimulation to the expiratory center on the floor of the medulla rostral to the obex. Injections of 30–60 mcg./Kg. 5-HT i. v. did not consistently block or lessen expiratory center stimulation; however, occasionally an inspiration did occur within the expected latency after injection. Complete apnea still persisted after carotid injections of serotonin. Pontine sectioned cats were made to inspire by intermittent tetanizing stimulation of the inspiratory center. Again injections of 5-HT, 30–60 mcg./Kg. i. v. did not block this form of artificial respiration, nor did microinjections of 0.005 ml. of 0.1% 5-HT at the site of medullary stimulation, but this effect could be blocked by microinjection of procaine 4%.

Figure 7 shows a series of tracings made from a cat which was caused to gasp by intermittent threshold tetanizing stimulation of either the left or right carotid body nerve. With injections of 5-HT, 30 mcg./Kg., this reflex gasp is seen in Fig. 7B to fatigue quite rapidly at the maximum of the depressor response. Switching from the left to the right carotid body nerve indicates that the bilateral reflex center rather than the nerve fiber is involved. The usual reflex gasps are either partially blocked in amplitude or completely prevented.

With the sectioning of all major nerves in the neck, the ganglionic chain below the superior cervical ganglia and the vago-sympathetic network, the blood pressure trace changes from a depressor to a

pressor reaction, Fig. 7C. With the injection of 5-HT, 30 mcg./Kg. i. v., the reflex gasps become inhibited at the maximum of the pressor response. In order to determine that this effect was not due to impulses conducted centrally by the neck nerves or in the spinal cord, a $C_6$ section was performed. Figure 7D shows the similar effect of the same dose of 5-HT on the induced reflex gasps thirty minutes following cord section. Finally in Fig. 7E, following sectioning of the brain stem above the medulla, the reflex gaspings are inhibited coincidentally with the injection of 5-HT. The head of the animal at this stage was connected to the trunk only by the carotid and vertebral arteries, the jugular vein, and the vertebral muscles. All other tissue in the neck had been ligated and severed, including both carotid sinus nerves, thus the only functional tissue would seem to be the cervical spinal cord and medulla connected efferently to the phrenic nerve. The afferent connections, the carotid body nerves, were spared since they were being stimulated for the gasp reflex.

## DISCUSSION

It has been stated previously that some of the confusion existing about the respiratory actions of 5-HT could be attributed to the lack of uniformity of description of the respiratory events. By not fully describing the respiratory reactions seen some of the more subtle actions of 5-HT have been overlooked. Using Fig. 2 upon which the three respiratory phases of 5-HT have been depicted it is possible to review previous findings in the light of some of the results presented in this paper.

The initial stimulation is the only respiratory action of 5-HT that is peculiar to the dog and not to cat, rabbit, rat, and guinea pig. Schneider and Yonkman (4) reported that double vagotomy abolished this primary respiratory stimulation by 5-HT and that the reflex receptor sites must then lie in the cardio-pulmonary region. Douglas and Toh (9), McCubbin, *et al.* (7), however, presented evidence that the origin of the respiratory stimulation is still functional in the vagotomized dog. McCubbin, *et al.*, disagree with Douglas and Toh, Heymans and van den Heuvel-Heymans (22) regarding the role of the carotid sinus and/or the carotid body. The latter two groups of investigators favor a central stimulatory action since stimulation persisted after sinus nerve sectioning in the vagotomized dog.

The same discrepancies and confusion exist for the primary apneic response seen in the cat. Reid and Rand (20) first reported that vagotomy eliminated this response. Comroe, *et al.* (2), Mott and Paintal (5), Paintal (6), Kottegoda and Mott (23), and Schneider and Yonkman (4) have all presented evidence that afferent fibers in the vagus are stimulated and sectioning or cooling the vagus to 1 or 2° abolishes apnea in most cats. Ginzel and Kottegoda (24) obtained evidence that baro- or chemoceptive stimulation caused an apnea which was abolished by sinus nerve sectioning. More recently Ginzel (10) has described apnea which persists after sinus nerve sectioning and can be elicited by the injection of small amounts of 5-HT into the subclavian artery near the vertebral artery.

Thus, for both the dog and the cat evidence has been obtained that 5-HT causes respiratory reactions at three different sites, cardio-pulmonary, sino-aortic, and central. As stated earlier, apnea in the cat is predominantly caused by a reflex arising from the lungs, the fibers having been identified by Paintal (6) as specific pulmonary deflator fibers. The initial gasps in dogs would appear largely due to stimulation of the chemoceptors of sino-aortic origin. It is proposed, however, that coincidental with the gasps there begins a true hyperpnea which may or may not be interrupted by apnea.

That two simultaneous forms of respiratory stimulation exist is suggested by the latent appearance of tachypnea with active expiration following one or more gasps. Inspection of published records (7, Fig. 3) of McCubbin, *et al.*, reveals a tachypnea but without a change in amplitude during which time the chemoceptors of the carotid bifurcation were removed from contact with circulating 5-HT.

The apneic response in dogs appears to have a dose-response relationship, for Douglas and Toh (9) report no apnea with 2–8 mcg./Kg. i. v. but Lucco (25) obtained a 20% response with 13 mcg./Kg. i. v.; Schneider and Yonkman (21) a 50% response with 50 mcg./Kg i. v.; and MacCanon and Horvath (8) report a 75% response when 2.5–5.0 mcg./Kg. was injected directly into the pulmonary artery.

Apnea in dogs appears to have an origin other than that seen in the cat. Intravenous injection of procaine prior to 5-HT abolishes apnea in the cat but not in dogs. Procaine does block the initial gasping and hyperpnea seen in the dog. In certain dogs there is close agreement between the dose-response and the time duration for both apnea and bronchoconstriction. The delayed appearance of apnea in dogs also suggests some other mechanism than that proposed for the cat. Preventing bronchoconstriction in certain dogs blocks the apneic response, Fig. 4. Schneider and Rinehart (16) although presenting no data regarding bronchoconstriction, describe the ability of reserpine to partially decrease apnea with better oxygenation. We have been able to repeat this experiment showing that reserpine has prevented bronchoconstriction. Prolongation of respiratory apnea in dogs following ganglionic blockade with pendiomide (21) could be accounted for by the inability of the sympathetic nervous system to antagonize bronchoconstriction. The slowly adapting bronchial receptors described by Widdicombe (26) can account for this reflex. Kottegoda and Mott (23) discuss this possibility with reference to the cat.

Most of the investigations to date have been concerned with the reflex nature of the respiratory action of 5-HT; however, most all investigators state that a residual central mechanism is involved. Stimulation as well as inhibition has been postulated. Marrazzi and Hart (27) first described a transitory inhibition of the ipsilateral transcallosal response of cortical neurons following injection of 5-HT into the carotid artery. Edisen (28) was able to show a slower but more prolonged depression of ipsilateral spinal mono- and multisynaptic conduction following large i. v. injections of 5-HT. More recently Koella, *et al.* (29), demonstrated both ipsilateral and contralateral inhibition of optically evoked cortical potentials resulting from carotid artery injection of small doses of 5-HT. Inhibition is more prominent when the carotid sinus is intact but there remains an

inhibition following sinus denervation which they attribute to a direct central effect. They ascribed the reflex and some of the direct inhibition as being mediated by the reticular core which relates directly with the decrease in facilitation of the knee jerk with respiratory inhibition (30).

Inhibition by 5-HT of the gasp reflex elicited by stimulating the carotid body nerve, Fig. 7, is an example of the central inhibition of a respiratory reflex. Partial confirmation of this action has been obtained by studying this same reflex in the recipient head of carotid cross-circulated dogs (31). Additional studies have been initiated to define the quantitative as well as qualitative role of 5-HT on central respiratory mechanisms.

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# The Quantitative Determination of Free Menthol in Peppermint Oil*

By MARTIN I. BLAKE†

**The free menthol content of peppermint oil is determined by refluxing a sample of oil with chloroacetyl chloride. Hydrogen chloride, a by-product of the reaction, is carried over into a solution of standard silver nitrate with the aid of nitrogen gas. Excess silver nitrate is determined with standard ammonium thiocyanate.**

THE OFFICIAL procedure for determining menthol in peppermint oil was developed by Power and Kleber (1) and first appeared in U. S. P. VIII. The method has been frequently criticized and, over the years, numerous modifications have appeared in the literature. These have been reviewed earlier (2, 3).

In general, methods proposed for the analysis of menthol take advantage of the reactivity of the hydroxyl group. In the official procedure for peppermint oil (4), the menthol is esterified with acetic anhydride and sodium acetate. Menthol content is evaluated by saponification of the ester with alcoholic potassium hydroxide. Christensen and Pennington (5) determined the menthol content of peppermint oil by acetylation with acetyl chloride. This reagent is also used for determining the hydroxyl number of fatty substances (6).

Methods for determining alcohol constituents in volatile oils are described by Guenther (7). Methods for evaluating alcohols in general, are reviewed by Mehlenbacher (8). These may be

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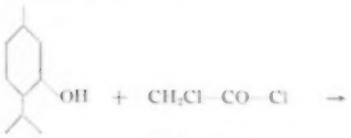
classified as acetylation (acetic anhydride or acetyl chloride), phthalation (phthalic anhydride), and formylation (aceto-formic acid reagent). Alcohols, including menthol, have been determined with lithium aluminum hydride by Lintner, Schleif, and Higuchi (9).

The present report describes a procedure for the estimation of menthol in peppermint oil by acylation with chloroacetyl chloride. The equation for the reaction is:




Menthol — Chloroacetyl Chloride

Menthyl Chloroacetate

The hydrogen chloride gas, a by-product of the reaction, is determined by the Volhard method.

## EXPERIMENTAL

**Apparatus.**—The arrangement of the apparatus is shown in Fig. 1. A standard nitrogen tank was used, and was equipped with a pressure gauge and reduction valve to permit control of the flow of nitrogen.

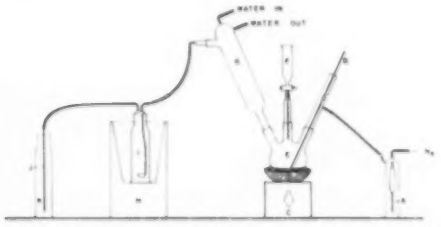



Fig. 1.—Apparatus for determining free menthol in peppermint oil: *A*, trap; *B*, thermometer; *C*, magnetic stirrer; *D*, heating mantle; *E*, reaction flask; *F*, filling tube; *G*, Friedrichs condenser; *H*, Dewar flask; *I*, vapor trap; *J*, measuring pipet; and *K*, graduated cylinder.

The outlet of the tank was connected to a trap (*A*) by tight fitting rubber tubing. The outlet of the trap was joined to a gas inlet of a 500-ml. three-necked standard-taper round-bottom flask (*E*). The reaction flask was fitted with a thermometer (*B*), a filling tube (*F*), and a Friedrichs condenser (*G*). A heating mantle (*D*) was set beneath the reaction flask and the entire system was mounted over a magnetic stirrer (*C*). The outlet of the condenser was fitted to a vapor trap (*I*) having a fritted disk sealed to the inner tube. The trap was placed in a Dewar flask (*H*) which was packed with crushed ice during a run. The trap outlet was joined to a small bore 1-ml. measuring pipet (*J*) by rubber tubing. The pipet was placed in a 50-ml. graduated cylinder (*K*) so that the tip of the pipet extended to about one-half inch from the base of the cylinder. All fittings were tightly secured to minimize the possibility of a gas leak.

**Procedure.**—A sample of pure menthol, 200–300 mg., accurately weighed, was dissolved in 20 ml. of toluene, and quantitatively transferred to the reaction flask. One gram of chloroacetyl chloride, dissolved in 20 ml. of toluene, was placed in the filling tube. Exactly 25 ml. of 0.1 *N* $AgNO_3$ solution was transferred by pipet to the 50-ml. cylinder. Distilled water was added to the 50-ml. mark. Nitrogen gas was passed through the system at a controlled rate of 120 bubbles per minute as noted in the graduated cylinder. The chloroacetyl chloride solution was added to the reaction flask and the heating unit was turned to "on" position. The solution was stirred gently during the heating period by means of a Teflon covered magnetic bar. The temperature of the reaction was maintained at 101–102°, by means of a rheostat for a period of one hour. After this time, the reaction was stopped and the contents of the 50-ml. cylinder were quantitatively transferred to a 250-ml. Erlenmeyer flask. Three milliliters of nitric acid and 3 ml. of nitrobenzene were added, followed by 2 ml. of ferric ammonium sulfate T. S. The excess silver nitrate was titrated with 0.1 *N* ammonium thiocyanate solution. A blank determination was run in a similar manner, but the menthol sample was omitted and a blank was run after each four determinations.

TABLE I.—ANALYSIS FOR FREE MENTHOL

| | | Peppermint Oil | | |
|---|---|---|---|---|
| No. | Pure Menthol Proposed Method, % | Proposed Method, % | Official Method, % | Method of Hamarneh, *et al.*, % |
| 1 | 101.78 | 50.93 | 51.20 | 50.99 |
| 2 | 100.80 | 50.90 | 50.85 | 50.76 |
| 3 | 100.74 | 50.26 | 50.78 | 50.49 |
| 4 | 100.22 | 50.01 | 50.02 | 50.45 |
| 5 | 99.95 | 49.76 | 49.51 | 50.30 |
| 6 | 99.88 | 49.46 | 48.96 | 50.26 |
| 7 | 99.67 | 49.10 | 48.76 | 50.00 |
| 8 | 98.97 | ... | ... | ... |
| Av. | 100.25 | 50.06 | 50.01 | 50.46 |
| Av. Dev. | 0.64 | 0.55 | 0.79 | 0.24 |

In the analysis of peppermint oil, samples weighing between 400 and 500 mg. (assuming about 50% menthol content) were employed. Samples were conveniently weighed by placing peppermint oil in a half-ounce dropper bottle. The entire system was weighed. Between 16 and 20 drops of oil were added to the reaction flask into which 40 ml. of toluene had previously been added. The dropper bottle was again weighed and from the difference in weight the sample weight was readily determined.

**Calculations.**—Menthol content was calculated from the expression:

$$\frac{\left[\begin{matrix}\text{Vol. 0.1 } N \text{ } NH_4SCN \\ \text{consumed in blank}\end{matrix} - \begin{matrix}\text{Vol. 0.1 } N \text{ } NH_4SCN \\ \text{consumed in run}\end{matrix}\right] \times 15.627 \times 100}{\text{Weight of sample in mg.}} = \% \text{ Menthol}$$

For a comparative study, peppermint oil was assayed by the U. S. P. method (4). Ester content and total menthol were determined. Free menthol was calculated from the results. Comparison was made with the results obtained by the method of Hamarneh, Blake, and Miller (3). All results are reported in Table I.

## DISCUSSION

This paper describes a convenient procedure for the determination of menthol, pure and in peppermint oil. The method is based upon an esterification procedure, the reaction commonly used for the estimation of menthol in peppermint oil. Instead of saponifying the ester, which is the usual procedure, the hydrogen chloride gas, a by-product of the reaction, is passed into a standard silver nitrate solution. This is effected with the aid of dry nitrogen gas which is passed through the system at a controlled rate. Excess silver nitrate is determined with standard ammonium thiocyanate solution.

The reagents employed in this procedure were selected because of their suitability for the conditions of the reaction. The temperature of the reaction was maintained at 101–102°, which was sufficiently high to insure completion of reaction in a reasonable time period, yet low enough that other products would not distil over. Since chloroacetyl chloride has a boiling point of 105°, whereas acetyl chloride boils at 51–52°, the former was selected as the acylating agent. Increase in boiling point is made at no significant expense to chemical reactivity. Toluene, with a boiling point of 111° served well as the reaction solvent. Menthol has a boiling point of 216°, far above the temperature of the reaction. To be certain that only the hydrogen chloride gas would be carried over, it was necessary to take additional precautions. The Friedrichs condenser with cold water passing through, served as a reflux condenser, permitting only gaseous products to pass through. In addition, the gases passed through a vapor trap maintained at a low temperature. A series of blank determinations showed a precision of better than 5 parts per 1,000.

The Volhard method was selected for the determination of the hydrogen chloride because the reaction could be followed conveniently by the nature and amount of the precipitate appearing in the graduated cylinder.

Preliminary investigation showed that the reaction, using sample weights indicated in this report, was complete within the one-hour time limit. On the average it required between four and five hours to conduct two runs and a blank. In routine analysis, a series of determinations may be conveniently effected by flushing the system with nitrogen gas prior to the introduction of a new sample and additional chloroacetyl chloride. However, for more accurate work it is suggested that a clean and dry reaction flask and vapor trap be installed for each run.

The results in Table I indicate that the proposed method gives results comparable to the official method and the chromatographic procedure reported earlier by Hamarneh, Blake, and Miller.

Work is currently in progress in this laboratory to apply this procedure to other alcohol-containing volatile oils.

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# Notes

## A Note on an Apparatus for Intracerebral Injections in Conscious Mice*

By J. VANECEK, V. KREBS†, E. SCHEER‡, and T. BIELEKE‡

RECENTLY intracerebral injections in mice have been successfully introduced as a method for studying the direct actions of drugs on the central nervous system with large groups of animals (1). Haley and McCormick (1) presented a detailed methodical account outlining the precise point and depth of injection, but a great degree of experimental skill was necessary for accuracy. Therefore, we tried to facilitate their technique by the following design (Fig. 1). It also helps to inject very small volumes of fluid accurately, without using specially accurate syringes.

A head holder (*A*) has been made from methyl methacrylate to assist in determining the point of injection. On its top, the midline and the points for injection in both ventricles are marked. The head of the mouse is placed in the holder so that the hind border connects the anterior base of the ears. The points for injection are then 2 mm. from the midline on both sides. This corresponds topographically to the point recommended for intracerebral injection in mice (1). The head is held in this position by a plate (*B*) with a coil spring (*C*).

In order to prevent the needle from penetrating into the mouth, the insertion depth is fixed at 2.5 mm. As the amount of injection fluid must not exceed 0.05 ml., a micrometer (*D*) operates the 1-ml. tuberculin syringe (*E*). This allows the precise administration of volumes of 0.001 ml.

Manipulation with the tuberculin syringe is facilitated with the micrometric screw (*F*), the weight is equalized by means of a counterweight (*G*), the thread of which is passed over a pulley (*H*). Two rods (*I*) maintain the same position in relation to the points for injection in the holder. The complete apparatus is attached to a stand (*J*).

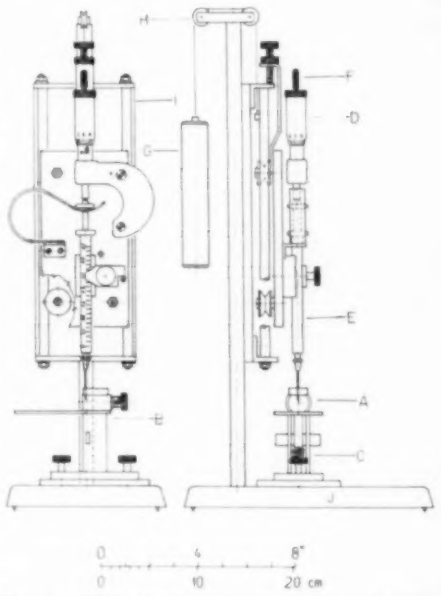


Fig. 1.—Scale drawing of intracerebral injection apparatus.

One hundred mice were injected with methylene blue, and about 85% of the injections were into the ventricle. It is hoped that this design may be of some use with the method of Haley and McCormick.

* Received November 2, 1959, from the Faculty of Pediatrics, Charles University, Prague, Czechoslovakia.
† Pharmacological Laboratory, Czechoslovakia Academy of Science.
‡ Institute for Corticovisceral Pathology and Therapy, Berlin, Germany.

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# A Note on the Correlation of *In Vivo* With *In Vitro* Disintegration Times of Enteric Coated Tablets*

By JOHN G. WAGNER

IN A PREVIOUS COMMUNICATION (1) the average disintegration time of enteric coated tablets in the intestine of the dog ($T_{vivo}$) was correlated with (*a*) the average disintegration time of the whole enteric coated tablet in artificial intestinal fluid pH 6.9, ($T_i$) and (*b*) the average disintegration time of the enteric coating only in the same fluid ($T_c$). The $T_c$ values were obtained by subtracting the average disintegration time of the corresponding subcoated tablet from the $T_i$ values. Linear relationships were obtained when $T_{vivo}$ was plotted against the logarithm of $T_i$ and when $T_{vivo}$ was plotted against the logarithm of $T_c$. The data used are shown in Table I.

The empirical relationships formerly reported are not supported by theoretical considerations. It has now been found that the above data may be plotted in a different manner to yield linear relationships which do have a theoretical basis. For reactions of order zero to two, the specific rate constant for the reaction is inversely proportional to the time required to reach a certain end point (2). If the reaction order for the disintegration process is from zero to two, then the specific rate constant will be inversely proportional to the disintegration time. Since the same end point was used both *in vivo* and *in vitro* (essentially complete disintegration of the enteric coated tablets), the reciprocal of the *in vivo* disintegration time may be plotted against the reciprocal of the *in vitro* disintegration time. The reciprocal, $1/T_{vivo}$, under the above conditions, will be directly proportional to the specific rate constant for the *in vivo* disintegration process and the reciprocal, $1/T_i$, will be directly proportional to the specific rate constant for the *in vitro* disintegration process.

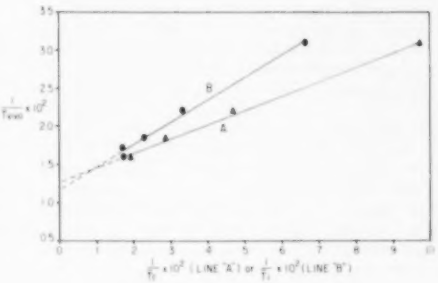


Fig. 1.—Curve *A* is a plot of the reciprocal of the average intestinal disintegration time in the dog against the reciprocal of the average disintegration time of the enteric coating in artificial intestinal fluid, pH 6.9. Curve *B* is a plot of the reciprocal of the average intestinal disintegration time in the dog against the reciprocal of the average disintegration time of the whole enteric coated tablet in artificial intestinal fluid, pH 6.9.

TABLE I.—IN VITRO AND IN VIVO DISINTEGRATION TIMES OF ENTERIC COATED TABLETS (TIME IN MINUTES)

| Lot | $T_i$ | $T_c$ | $T_{vivo}$ | Type of Enteric Coating |
|---|---|---|---|---|
| I | 15.1 | 10.3 | 32.2 | Styrene-maleic acid copolymer, dibutyl phthalate, and talc |
| II | 30.1 | 21.3 | 45.2 | Hydrolyzed resin SC-2, dibutyl phthalate, and talc |
| III | 43.8 | 35.0 | 54.0 | Cellulose acetate phthalate, propylene glycol, Span 80, and talc |
| IV | 58.2 | 52.4 | 62.5 | Starch acetate phthalate, propylene glycol, Span 80, and talc |
| II[a] | 59.8 | ... | 58.3 | |

[a] Lot II stored for one month at 47°.

Figure 1 shows a plot of $1/T_{vivo}$ against $1/T_c$ (line *A*) and a plot of $1/T_{vivo}$ against $1/T_i$ (line *B*). The correction coefficient for line *A* is 0.998 and that for line *B* is 0.996. Both correlations are highly significant. The equations for the least squares lines are

$$1/T_{vivo} = 0.190\,(1/T_c) + 0.0128 \text{ (line } A\text{)} \quad \text{(Eq. 1)}$$

$$1/T_{vivo} = 0.294\,(1/T_i) + 0.0118 \text{ (line } B\text{)} \quad \text{(Eq. 2)}$$

Assuming the reaction order is between zero and two and is the same *in vivo* as *in vitro*, Eq. 2 indicates that the rate constant for the disintegration process in the dog's intestine is only approximately one-third of the rate constant for the disintegration process in pH 6.9 intestinal fluid when the whole enteric coated tablet is considered.

Equations 1 and 2 are only valid over the range of disintegration times studied. Equation 2, if utilized outside this range, indicates that as $T_i$ gets very large, or $1/T_i$ approaches zero, then $1/T_{vivo}$ approaches 0.0118, or $T_{vivo}$ approaches 1/0.0118 or eighty-five minutes. However, it is known from our investigation of lot V in the dog (1) and the work of Chapman, *et al.* (3, 4), in human subjects that tablets which have very large disintegration times may not disintegrate completely, or at all, in the intestines. Hence, one can assume that beyond a certain $T_i$ value, greater than sixty minutes, the enteric coated tablets will not disintegrate completely in the intestine.

* Received August 13, 1959, from the Upjohn Co., Kalamazoo, Mich.

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# A Note on the Preparation of Saturated Solutions at Constant Temperatures*

By J. A. WOOD, L. WAIT RISING, AND NATHAN A. HALL

**An easily constructed apparatus for the uniform agitation of solute-solvent combinations is described. It can be used effectively in many laboratory mixing problems.**

IN CONNECTION with our studies of the solubilization of certain chemicals by surfactants, the construction of an apparatus for uniform agitation of the solute-solvent combination became necessary. By a relatively simple adaptation of materials common to most pharmaceutical research or control laboratories, we have been able to fashion a suitable instrument, the detailed construction of which may be of interest to others faced with a similar problem.

The arrangement, illustrated in Fig. 1a and the photograph, Fig. 1b, makes use of the Vanderkamp tablet disintegration tester (Stoll-Gershberg Type)[1] (*a*). The apparatus submits the solute-solvent mixtures to a continuous rocking motion which provides the necessary agitation. The vertical shaft (*b*) is moved up and down within its supporting column in a stroke of 5.5 cm. by a special steel-geared reduction motor and an eccentric cam at a fixed speed of 30 cycles per minute. This speed is suitable for limpid and viscid mixtures.

In the assembled disintegration apparatus a basket-rack assembly is fastened to the end of the supporting rod (*d*) and thereby hangs from the horizontal arm (*c*). The basket-rack assembly is removed and its supporting rod (*d*) is connected to an aluminum rod (*f*), $^3/_8$ inch diameter, by means of a piece of thick-walled rubber tubing (*e*). The terminal $^3/_8$ inch of rod (*f*) is filed flat on two sides and fitted into a slot cut into the end of the tilting arm (*h*). A hole is drilled through the two pieces of rod and a pin (*g*) inserted to form a pivot joint.

The tilting arm (*h*) is a Flexaframe rod (Fisher Scientific Co., Catalog No. 14-666-10) rigidly connected to another Flexaframe rod whose end is visible at point (*i*) by a Flexaframe connector (*i*) (Fisher Scientific Co., Catalog No. 14-666-20). Fastened to the rod visible at (*j*) is a wooden platform (*l*), $^1/_2 \times 3 \times 6$ inches, upon which is mounted the solution bottles. The wooden platform is fastened to its mounting rod (*j*) by means of two aluminum straps placed over the rod and screwed to the wood. The straps used are those which an electrician uses to strap conduit to rafters or walls. The solution bottles (*m*), only one visible in drawing, are mounted on the wooden table by means of two elastic tapes (*n*). These secure the bottles but permit easy mounting and removal.

The mounting rod visible at (*j*) is supported at each end by Flexaframe connectors (*k*), only one visible in drawing, which are in turn fastened to two supporting rods (*o*), only one visible in drawing. The lock-screw is removed from connectors (*k*) so that the rod at (*j*) is free to rotate. The two

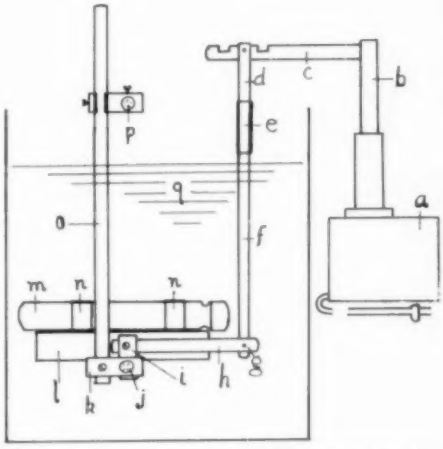


Fig. 1a.—Apparatus for preparing saturated solutions.

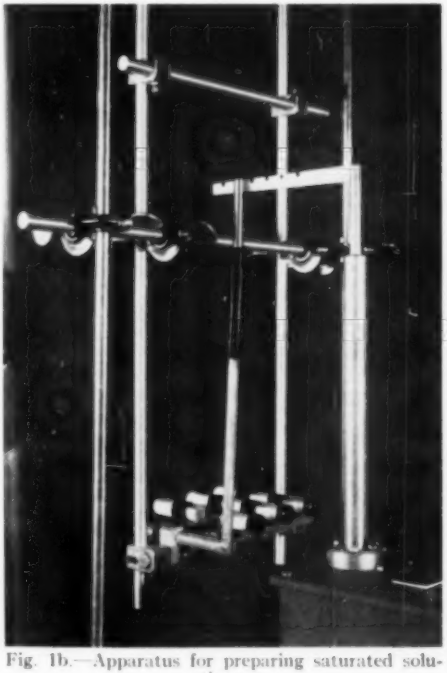

Fig. 1b.—Apparatus for preparing saturated solutions.

* Received August 17, 1959, from the University of Washington, College of Pharmacy, Seattle 5.

[1] Purchased from Parlam Corporation, 340 Canal St., New York 13, N. Y.

support rods (*o*) are maintained at a fixed distance apart by a cross-member rigidly connected near the top of the rods (*p*). The cross member visible at (*p*) is in turn clamped to mounting rods secured to the water bath. This arrangement of supports and connectors serves as a mounting device to suspend the solution bottles in a constant-temperature water bath and as bearings for the operation of the rod supporting the bottles. The water (*q*) provides adequate lubrication for the rod ends (*j*) in the connector bearings. The effective length of the tilting arm (*h*) is 6.3 cm., and it is operated by a vertical stroke of 5.5 cm., see above; therefore, the angle of tilt through which the solution bottles move is about 50°, sufficient to provide good agitation.

When agitation has continued sufficiently long to saturate the solvent with solute the rod (*d*) is lifted off the supporting arm (*c*) and may be secured by a suitably mounted hook in such a position that the solution bottles are in an upright position. The excess solute is allowed to settle or may be centrifuged to produce a clear supernatant liquid for analysis.

# Book Notices

*Treatise on Analytical Chemistry.* Part 1: Theory and Practice. Vol. I. Edited by I. M. KOLTHOFF and PHILIP J. ELVING. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. xxvi + 809 pp. 16 x 23.5 cm. Price single $17.50, subscription, $15.

This is the first volume of the first of three parts of a comprehensive and systematic treatise on all aspects of classical and modern analytical chemistry, covering the scientific and instrumental fundamentals of analytical methods by critical selection and interpretation of methods and procedures for inorganic and organic compounds. Methods for investigation and evaluation of the properties and composition of commercial products will be included. The first four chapters (Section A) of volume 1 take up "Analytical chemistry: its objectives, functions, and limitations." Section B, chapters 5–19, covers "Application of chemical principles." Each chapter is a contribution by an authoritative worker in the field discussed. References are given at the end of each chapter. Volume 1 does not contain an index but a detailed table of contents is provided. The Treatise is not intended to replace the great number of existing and exhaustive monographs on specific subjects, but rather to serve as an introduction and guide to the efficient utilization of specialized monographs. Volume 1, part 1 is a good beginning.

*Husa's Pharmaceutical Dispensing.* Edited by ERIC W. MARTIN. Mack Publishing Co., 20th and Northampton Sts., Easton, Pa., 1959. ix + 729 pp. 18 x 25 cm. Price $12.

This textbook has been completely rewritten, with twenty-six authors contributing different chapters. Its style and content are as up-to-date as possible. Particularly interesting is the treatment of "Incompatabilities" in three chapters covering 231 pages. A section on compatibilities and incompatibilities of certain manufactured products is included. The book is directed not only toward helping the individual who compounds prescriptions, but also to the future pharmacists' knowledge about commercial compounding practices as they affect prescription writing by the physician.

*Curare and Curare-Like Agents.* Edited by D. BOVET, F. BOVET-NITTI, and G. B. MARINI-BETTÒLO. Elsevier Publishing Co., Amsterdam, 1959. Distributors for the United States, D. Van Nostrand Co., 120 Alexander St., Princeton, N. J. xi + 478 pp. 16.5 x 24 cm. Price $15.75.

This book is a comprehensive monograph on curare and curare-like agents in which authoritative contributors have written on subjects which are grouped under the following main chapter headings: Ethnographic problems connected with the preparation and use of curare by South American Indians, South American Loganiaceae and menispermaceae as a source of curarizing alkaloids, The Chemistry of naturally occurring curarizing alkaloids, Pharmacological and physiological aspects of curare and curare-like drugs, and Clinical applications of curarizing agents. References are given at the end of each section. A subject index is appended.

*Pain and Itch: Nervous Mechanisms.* By Ciba Foundation Study Group No. 1. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. vii + 120 pp. 12 x 18.5 cm.

A compilation of the papers and discussions at the symposium of the Ciba Foundation study group No. 1, March 10, 1959. References and an index are appended.

*Analytical Chemistry of Titanium Metals and Compounds.* Chemical Analysis. Vol. 9. By MAURICE CODELL. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. xiii + 378 pp. 15 x 23 cm. Price $12

This is the ninth volume of a series of monographs on analytical chemistry and its applications edited by P. J. Elving and I. M. Kolthoff. The author of this monograph, M. Codell, has gathered information from reliable authorities in the special field covered. The book is designed to provide analytical chemists with the information required to analyze any titanium-containing material. All references as well as author and subject indexes are appended.

*Industrial Gums.* Polysaccharides and Their Derivatives. Edited by ROY L. WHISTLER and JAMES N. BEMILLER. Academic Press, 111 Fifth Ave., New York 3, N. Y., 1959. xi + 766 pp. 15 x 23 cm. Price $25.

This book presents practical information on industrial gums for the use of researchers, chemical engineers, and technical sales personnel. Discussions cover factors influencing gum costs and applications and the individual gums from agar and gum arabic, through the dextrans and bean and seed gums as well as the cellulose derivatives, to tamarind and ti. A good index is appended.

*Clark's Applied Pharmacology.* 9th ed. By ANDREW WILSON and H. O. SCHILD. Medical Book Dept., Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. xii + 750 pp. 14.5 x 22 cm. Price $10.

A revised edition with new chapters on the pharmacology of tuberculosis and on psycho-pharmacology, the ninth edition of this textbook correlates the science of pharmacology with the art of therapeutics; focusing on the problems of clinical medicine and the action of drugs upon the functions of the body. The book is designed to stimulate a critical approach to the problems in the complex field of drug therapy.

*Atlas of Human Anatomy.* 5th ed. By FRANZ FROHSE, MAX BRÖDEL, and LEON SCHLOSSBERG. Barnes & Noble, Inc., 105 Fifth Ave., New York 3, N. Y., 1959. x + 180 pp. 13.5 x 21 cm. Price, Paperbound $2.95, Cloth $4.50.

This pocket size edition of the Atlas includes page size color reproductions of the Frohse-Brödel wall charts. Also Schlossberg's supplementary charts and an explanatory text. Other color charts (endocrine system) and line drawings are included. An index is appended.

*Methoden der organischen Chemie (Houben-Weyl).* Band 1/2, Allgemeine Laboratoriumspraxis II. Georg Thieme Verlag, Herdweg 63, Stuttgart, N. Germany, 1959. xlv + 1017 pp. 17 x 25.5 cm. Price DM 176.40.

The latest volume of this comprehensive treatise (in German) of the methods and procedures in organic chemistry is the second part of the portion devoted to general laboratory practice. The volumes in this treatise have not appeared consecutively. The publication notice of Band XI/2 on nitrogen compounds, the immediately preceding volume, appeared in THIS JOURNAL, **48,** 363(1959). The first part of the present volume deals with methods of comminution and division of materials. The major portion (573 pages) is devoted to general laboratory procedures: from "measurement of liquids by gravity and pump methods" to "heat and cold; measurement, registering, and regulating temperatures." All the sections are liberally illustrated with photographs and diagrams of the apparatus mentioned. The third portion of the book discusses accessory aids in laboratory technique. A final section covers accident prevention in the laboratory, and concludes with a discussion on carcinogenic substances. Author and subject indexes are included.

*A History of the American Dental Association.* By ROBERT W. MCCLUGGAGE. American Dental Association. 222 East Superior St., Chicago 11, Ill., 1959. 520 pp. 16.5 x 23.5 cm. Price $8, special red leather binding $12.50.

The stated aim of this book is not to present the history of the American Dental Association as a series of dates, marshalled in chronology and illustrated by faded photographs of bearded men, but rather to present its history against the social and professional background of the times. The story is presented in proper perspective, since the personalities, crises, failures, and achievements which are described integrate this study of dentistry in general, and its professional society in particular, with the overall historical period. The pertinent facts are intertwined with an unusual insight into the various patterns of dental education, research, and practice over the years, which makes reading of the book flow quite easily and naturally.

It is immediately apparent that, in spite of its 520 pages, as much condensation as possible has been achieved. The result is a rapidly moving book with all the necessary facts described as briefly as possible. The historical significance, however, does not suffer by this, but in fact is further enhanced by the excellent documentation with references and explanatory notes grouped by chapters and collected at the end of the book.

While the prime purpose of this publication is to present a history of the ADA, a much broader result has been achieved. The book might equally be regarded as a history of American dentistry—its evaluation, professionalism, and heritage.

*Précis de Chimie Biologique.* Vol. 1. By J. E. COURTOIS and R. PERLÈS. Masson et Cie, 120, boulevard Saint-Germain, Paris, 6e, France, 1959. viii + 453 pp. 16 x 21 cm. Price 3,400 fr.

This book (in French) is a concise textbook on biochemistry. It is one of a series of textbooks designed particularly for pharmacy students. The series is appearing under the general title "Collection de Précis de Pharmacie," edited by M.-M. Janot.

*Précis de Pharmacodynamie.* By G. VALETTE. Masson et Cie., 120, boulevard Saint-Germain Paris, 6e, France, 1959. viii + 519 pp. 16 x 21 cm. Price 4,200 fr.

This is the third book (in French) in the series "Collection de Précis de Pharmacie," edited by M.-M. Janot for use as textbooks for students of pharmacy and related sciences. It follows the style adopted for this series in that it is a concise presentation of the action of medicaments, or more generally, chemical substances on the living organism.

*Soil, Grass and Cancer.* By ANDRÉ VOISIN. Philosophical Library, Inc., 15 E. 40th St., New York 16, N. Y., 1959. xvii + 302 pp. 13.5 x 21.5 cm. Price $15.

This book presents the author's views on the relation between the manipulation of the soil and its products and human nutrition. Voisin holds that cancer appears less frequently in dwellers on calcareous areas of different types of soils.

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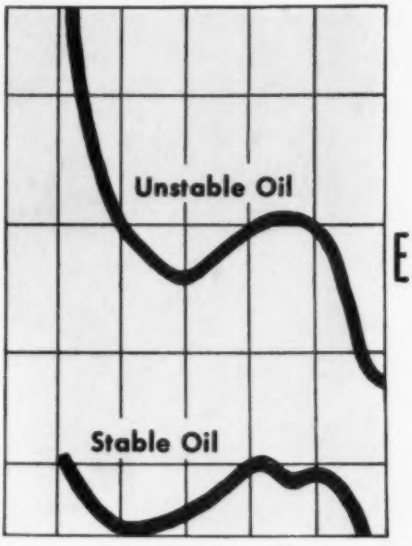

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